J. Afr. Ass. Physiol. Sci 5 (1): 45-57, July 2017

Journal of African Association of Physiological Sciences

Official Publication of the African Association of Physiological Sciences
http://www.jaaps.aapsnet.org

Research Article

Influence of genistein aglycone on some male reproductive functions in pubertal *Holtzman* rats

F.O. Awobajo [a,b]*, O.T. Oyelowo[b], K. Dumasia [a], and N.H. Balasinor[a]

[a]Department of Neuroendocrinology, National Institute for Research in Reproductive Health, Parel-400012, Mumbai, India and [b]Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, Nigeria

Keywords:
Acrosome reaction, computer assisted sperm analyser, male potency, fertility index, Genistein.

ABSTRACT

Background: Genistein, a phytoestrogen found in abundance in soya has been known to adversely influence male reproductive system. The effects of genistein on some male reproductive functions were investigated in pubertal laboratory rats. **Methods:** Male *Holtzman* rats, 70-75 days old and weighing 200-250g were used for the study. They were grouped into five groups of ten rats each. Group 1 (control) received distilled water. Groups 2, 3, 4, and 5 were administered orally with 0.5, 1, 2, 4 mg/ kg body weight of genistein respectively for a period of 60 days. Daily feed consumption and final body weight were recorded. Sperm count and motility were analysed along with the serum testosterone, estradiol and leptin levels. Acrosome reaction (AcR) was assessed using PSA-FITC with calcium and progesterone as stimulants. Male potency and fertility index were also calculated. **Results:** There was significant decrease in feed consumption in the 2 and 4 mg/kg genistein groups within the first 15 days of the experiment with a corresponding decrease in final body weights in 0.5, 2 and 4 mg/kg groups. A significant decrease was recorded in the right and the left absolute testicular weights in 0.5, 2 and 4 mg/kg groups while the right and left absolute epididymal weight and the prostate gland were significantly reduced in 2 and 4 mg/kg groups. The result showed a significant decrease and increase in serum testosterone level in 0.5 and 1 mg/kg groups respectively. Estradiol level was significantly reduced in all the genistein treated groups. Serum leptin was significantly increased in 1 mg/kg group. Sperm count was significantly reduced in 4 mg/kg group while sperm motility was reduced in the 2, and 4 mg/kg groups. Sperm track speed; lateral amplitude and elongation were significantly increased in all the genistein groups. Sperm path velocity was increased in all genistein groups except in the 4 mg/kg group. Progesterone stimulated AcR resulted in largely intact acrosome in all genistein group while calcium significantly increased percentage of reacted acrosome in 0.5 mg/kg group. Combined stimulation with progesterone and calcium increased AcR when compared with either of the stimulators alone. Potency and fertility index were significantly reduced in both 2 and 4 mg/kg groups while days of cohabitation before successful mating were increased in the 2 and 4 mg/kg groups. Ploidy analysis showed a significant increase and decrease in the population of elongating spermatid (HCI) and round spermatid (IC) in 2 and 4 mg/kg respectively. Pre-leptotene spermatocytes and primary spermatozoa population were significantly decreased in 4 mg/kg group. **Conclusion:** Oral administration of genistein to pubertal *Holtzman* male rats adversely influenced some important male reproductive functions.

INTRODUCTION

Male reproduction is known to be highly susceptible to environmental factors that can disrupt spermatogenesis or adversely affect testosterone synthesis. The overbearing influences of testosterone, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) in the regulation of male fertility cannot be over emphasized. However, advance in biomedical research have shed more light on the possible roles of estrogen on the functionality of the male reproductive system (Dumasia *et al*., 2015; Cooke *et al*., 2017). Genistein (5, 7-dihydroxy-3-(4-hydroxyphenyl) chromen-4-one) is an estrogenic isoflavone found in leguminous plants (Breinholt *et al*., 2000; Jefferson *et al*., 2012) and is

*Address for correspondence:
E-mail: funmi_bajo@yahoo.com

particularly abundant in diets containing soya. It has been reported that humans are exposed at a dietary level as high as 2 mg/kg bodyweight/day and infants as high as 1 mg/kg body weight/day (Setchell *et al*., 1998). Genistein has tyrosine kinase inhibitory activity (Akinyama *et al*., 1987) as well as radio-protective properties (Landauer *et al*., 2003). Genistein gained prominence because of its estrogenic activity with the potential of serving as hormonal replacement therapy in menopausal women (Whitten and Naftolin, 1998; Bouker and Hilakivi-Clarke, 2000) in addition to its potential as a possible anticancer agent (Hsieh *et al*., 1998; Tian *et al*., 2014). Genistein has been reported to adversely affect reproductive development in the foetus exposed *in-utero* (Jefferson *et al*., 2012).

Studies in our laboratory on the effect of administration of genistein to pregnant rats showed notable adverse effect on fertilization of oocyte, increased resorption and reduction in fetal and placental weights especially at a dose of 2 mg/kg body weight when administered for 15 days prior to pregnancy (Awobajo *et al*., 2013). There are also reports on the possible influence of genistein when exposed in-utero or during lactation on postnatal development in rodents (Lian-Dong *et al*., 2013) as well as effects of such exposures on male reproductive variances (Wisniewski *et al*., 2003). Some authors however reported no anomalies in exposed male rodents (Faqi et al., 2004; Jung *et al*., 2004). Apart from sperm concentration and motility which are routinely used in assessment of male reproductive functions and fertility, Acrosome Reaction (AcR) evaluation has become a major requirement in such assessment in relation to the fertilization of oocytes. Acrosome reaction is an exocytotic event that is absolutely required for the fusion of the spermatozoa with the oocyte released at ovulation in mammals. This process empowers the spermatozoa to pass through the zona pellucida for onward fusion with the oocyte. A zona glycoprotein ZP3 found on the oocyte has been shown as the putative primary sperm receptor responsible for inducing a signal transduction cascade leading to the AcR in the sperm cell (Gupta and Bhandari, 2011). Endogenous progesterone found along the uterine wall is known to trigger the AcR via its influence on the increasing influx of calcium ion (Calogero *et al*., 2000). Sperm capacitation; a biochemical change in the membrane of the sperm cell that makes AcR possible is a tyrosine phosphorylation related process with the possibility of being inhibited by genistein a known tyrosine kinase inhibitor (Leyton, 1992). The estrogenic activities of genistein may also influence male reproductive functions (Montani *et al*., 2008). In a study carried out by Opalka *et al.* (2004) in roosters, Genistein had a dose-dependent reduction effect on Leydig cell secretion of testosterone in the presence of luteinizing hormone. Furthermore, genistein negatively affected acrosome reaction and sperm capacitation in bull and rats in a dose-dependent manner (Kumi-Diaka and Townsend, 2003). Other studies have shown adverse effects of genistein on the development of reproductive system before and after puberty in animals (Nago *et al*., 2001; Wisniewski *et al*., 2003; Lee *et al*., 2004). In this study, we chose four doses of 0.5, 1, 2, 4 mg/kg/day of genistein which broadly covers the average exposure level of (4 mg/kg genistein) in an adult (Setchell *et al*., 1998). This study intends to elucidate some of the mechanisms via which genistein produces adverse effects on male reproductive functions in pubertal male *Holtzman* rats.

MATERIALS AND METHODS

Genistein preparation and administration

Genistein (purity, 99%) was obtained from Sigma-Aldrich U.S.A. Genistein was prepared with distilled water and mixed vigorously prior to use. All animals received humane care as outlined by the 'Guide for the care and use of animals' as approved by the Institutional ethical committee, National Institute for Research in Reproductive Health, India with approval number IAEC Approval no:14/14. This is also in accordance with the international standard on care and the use of animal for experimental purposes (NACLAR 2004).

Animal treatment

Pubertal male *Holtzman* rats were used for this study. The male *Holtzman* rats aged 70 - 75 days and weighing 200 - 250 g were used for the study. The animal facility was maintained under controlled room temperature of 22 ± 2 ^{o}C, relative humidity of 55 – 65 % and artificial illumination with a 14 hour light – 10 hour dark cycle. The rats had access to pelleted rat chow and water *ad libitum*. There were five groups of ten rats each. Group 1 served as control and received distilled water which was the vehicle for genistein, while groups 2, 3, 4 and 5 were administered with 0.5, 1, 2 and 4 mg/kg body weight respectively. All administration was carried out through oral gavage for 60 days. Daily feed consumption was calculated by subtracting weight of left over feed every morning from the weight of feed supplied the previous night. Animals were stunned before been euthanized by cervical dislocation to collect blood samples uncontaminated by chemical agents (Close *et. al*., 1996; The University of Texas 2013). The various reproductive organs including testes, epididymis, seminal vesicle and prostate were carefully dissected out and weighed.

Mating study

At the end of the sixty days' exposure, some of the animals in the different groups were separated and

cohabited with mature virgin female rats that were at proestrus stage. Mating was done at a ratio of 2 female rats to one male rat. Daily vaginal smear was carefully prepared from each female rat with the aid of a warm normal saline, glass slides and a Pasteur pipette that has a smooth rubber fitted to its tip. Presence of sperm cells was confirmed by observing the smeared glass slides under the light microscope. The number of days of cohabitation before a successful mating was established as signified by the presence of a vaginal sperm plug or the presence of sperm cells in the vaginal smear. This was recorded for each male rat. Potency/Mating index was calculated as: Mating index = $\frac{No\ of\ female\ rats\ that\ mated}{No\ of\ female\ rats\ Cohabited}\ x\ 100$, (Dumasia *et al*., 2015) while fertility index was expressed as the ratio of the number of implantation sites to the number of corpora lutea (Gill-Sharma *et al*., 2001). Potency is a measure of the ability of male to successfully inseminate a female rat.

Feed Consumption, reproductive organ weight and testicular cell counts

Daily feed consumption was calculated by subtracting the weight of the rat pellets left overnight from the amount of feed supplied to the animals the previous morning. The amount consumed by the group was divided by the number of rats in the cage. The reproductive organs were carefully dissected and weighed on the warm stage of a precision digital milligram scale balance (Mettler-Toledo Switzerland**)**. The relative weights of the testis, epididymis and prostate were determined by dividing the weight of the animals' body and expressing it in percentage. The testicular cell count was also assessed by mincing the testis with the aid of sterile scissors and thereafter passing it through a 40 mm cell strainer. 0.1 µl of the effluent was serially diluted and the cell counted with the aid of a hemocytometer under the x400 magnification with a compound microscope. Final cell count was calculated with consideration for the volume of dilution.

Hormonal analysis

Blood samples were collected into sterile sample bottles and allowed to clot. The blood samples were centrifuged at 7000 rpm for 15 minutes and the serum was carefully collected into sterile eppendorf tubes and stored frozen at - 80 ^{o}C. Serum testosterone, estradiol and leptin levels were measured using ELISA assay kits (Sigma Aldrich, USA) according to the manufacturer's protocol.

Sperm motility and motion analysis

The Caudal Epididymis (CE) was carefully separated from the caput, weighed on the warm stage of a precision digital milligram scale balance (Mettler-Toledo Switzerland) and its volume was determined by submerging it in warm (37 °C) 5 ml Dulbecco's Modified Eagle Media (DMEM) supplemented with Bovine Serum Albumin (BSA). All procedures were carried out at room temperature while all instruments and petri dishes were maintained at 37 °C. Thereafter, 5-7 radial cut were made on the CE using a warm pair of scissors to release the sperm cells into the warm DMEM-BSA in a warm petri dish. The released sperm cells were incubated at 37 °C for 5 minutes. Afterwards 10 µL of sperm suspension was placed into an 80-µm-deep, 2X-Cel disposable sperm analysis chamber (Hamilton-Thorne Research, Beverly, MA, USA). The device was placed on the heated stage (37 °C) of a Computer-Aided Sperm Analysis (CASA) integrated visual optical system (Hamilton-Thorne Research USA). The acquisition parameters and rates used for analysis were those of standard rat analysis setup 1 (frame rate, 60 Hz; frames acquired, 30; minimum cell size, 7 pixels; minimum contrast, 15; brightness, 2,500). Sperm were considered motile if the Average Path Velocity (APV) exceeded 20 µm/sec; they were considered progressively motile when APV exceeded 50 µm/sec and Straightness of Tajectory (STR) exceeded 80%. A minimum of 200 motile sperm cells were analysed from each animal for the following variables: percentage of motile sperm, percentage of progressively motile sperm, APV (micrometer per second), Straight-Line Velocity (VSL; micrometer per second), curvilinear velocity (VCL; micrometer per second), lateral head displacement (ALH; micrometer), beat cross frequency (BCF; Hz), straightness STR (%), and linearity of trajectory (LIN; %).

Acrosome reaction

Acrosome reaction "AcR" was determined using progesterone (100 µl of 1 mM of progesterone prepared in DMSO) and calcium ionophore (2 µl of 8 µM of calcium ionophore in DMSO) as capacitant. The set up involved addition of progesterone and/or calcium ionophore or combination of both progesterone and calcium ionophore to 100 µl sperm suspension in an Eppendorf tube with a control setup having sperm suspension without any of the capacitant. The set up was incubated at 37 ^{0}C in 5 % CO_2 for 1 hour in the case of calcium ionophore and 2 hours in the case of samples capacitated with progesterone. After the incubation period allowed, sperm smear was prepared on a polyglysine coated glass slide and allowed to dry at room temperature. The dry slides were fixed in chilled acetone at -20 ^{o}C for 20 minutes and thereafter dried at room temperature. The dried slides were washed twice with Phosphate Buffer Solution (PBS). The slides were incubated with Pisium Sativum

Agglutinin- Fluorescein isothiocyanate (PSA-FITC) for 1 hour at dark room temperature (1:500 dilutions in PBS) and thereafter incubated with propidium iodide (1 ml/10 ml PBS) by flowing solution on slides for 20 minutes. Slides were washed with PBS-Tween 20 twice and dried in the dark at room temperature. The slides were mounted with cover slip using DPX mountant (Qualigens, fine chemical India) and thereafter observed for acrosome reaction under Epi-fluorescent microscope (Axioskop Carl Zeiss, Germany). At least 200 cells were counted for each slide and scored as either acrosome reacted (Rx), partial reaction (PRx) or intact acrosome (IT).

Epididymal Sperm Concentration

Epididymal sperm concentration was determined using aliquot part of solution prepared for acrosome reaction above. Briefly, the epididymis volume was measured by immersing it in 5 ml of DMSO at room temperature. The epididymis in the DMSO was poured into a warm petri dish and carefully lacerated and mashed out to release all the sperm cells. Thereafter, 0.1 µl of the aliquot was pipetted into another Eppendorf tube containing 2 ml of DMSO. Sperm count was carried out using the Neubauer cell counter with its counting chamber charged with 0.1 µl. of the diluted sperm sample and observed at x400 magnification under a light microscope (Badkoobeh *et al*., 2013). Adjustment was made for the volume used for dilution and the final concentration calculated for the volume of the epididymis.

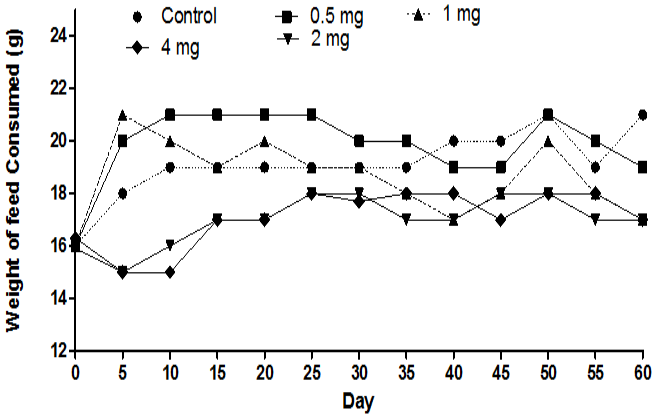

Fig. 1. Feed consumed in genistein treated rats compared with control

Ploidy analysis using flow cytometry analysis

Testicular cells prepared from control rats and rats treated with genistein (2 or 4 mg/ kg for 60 days) were processed for flow cytometry analysis. Briefly, the male rats were sacrificed by cervical dislocation and the testes removed and washed in cold PBS.

Testicular cell suspension was prepared by passing the seminiferous tubules through 100 µm and 40 µm cell strainers in PBS. The concentration of the testicular cell samples was determined. Aliquots of 1 x 10^6 cells/ml were fixed using cold 70 % alcohol and stored overnight at 4 ^{0}C. The fixed cells were washed twice in PBS and treated with 0.2 mg/ml RNase (Ameresco, Solon, OH, USA) and stained with 50 mg/ml Propidium Iodide (PI) (Calbiochem, La Jolla, CA, USA) for 20 minutes. The testicular cells samples were passed through a 40 mm cell strainer and a total of 10,000 ungated events were recorded for each experiment on BD-flow cytometer with Argon laser detector (Becton Dickinson; San Jose, CA, USA) and analysed using FACS Diva 6.1.3 software (BD, San Jose, CA, USA).

Statistical analysis

All data were analysed with Graph pad prism5 using ANOVA and post hoc analysis using Duncan post hoc test. The results were displayed as mean ± SEM and the level of significance was placed at $p \leq 0.05$. Bar chart and line graphs were also used for graphical presentation.

RESULTS

Feed consumption, relative weight of some male reproductive organs and testicular cell count from control and genistein exposed

Daily feed consumption declined initially in the 2 and 4 mg/kg groups till around the 15th day of experiment. Thereafter the daily feed consumption was no longer significantly different between all the genistein treated group compared with control. The final body weights of animals treated with 0.5 mg, 2 mg and 4 mg/kg of genistein were significantly lowered compared with control (Fig 2; panel K). Significant reductions in the absolute weights of the testes were recorded in all rats treated with genistein at a dose of 0.5 mg, 2mg and 4 mg/kg body weight. Significant increase was however, recorded in the relative testicular weight at the 1 mg/kg, 2 mg/kg and 4 mg/kg (Fig 3; panel Q). However, this significant increase in testicular weight occurred in consonance with a significant decrease in testicular cell count in the 2 mg and 4 mg/kg group. The absolute weights of the epididymis were significantly reduced in 2 and 4 mg/kg groups. This gave a significant increase in the relative weights of the right epididymis in all the 1 mg/kg, 2 mg/kg and 4 mg/kg genistein groups and the weight of the left epididymis of 2 mg and 4 mg/kg when compared with the control. There was also a significant reduction in the absolute prostate weights in the 1 mg, 2 mg and 4 mg/kg genistein groups. There was however, no difference in the relative prostate weights among the genistein treated groups compared with control. A significant reduction in the absolute and relative weight of the cowper's

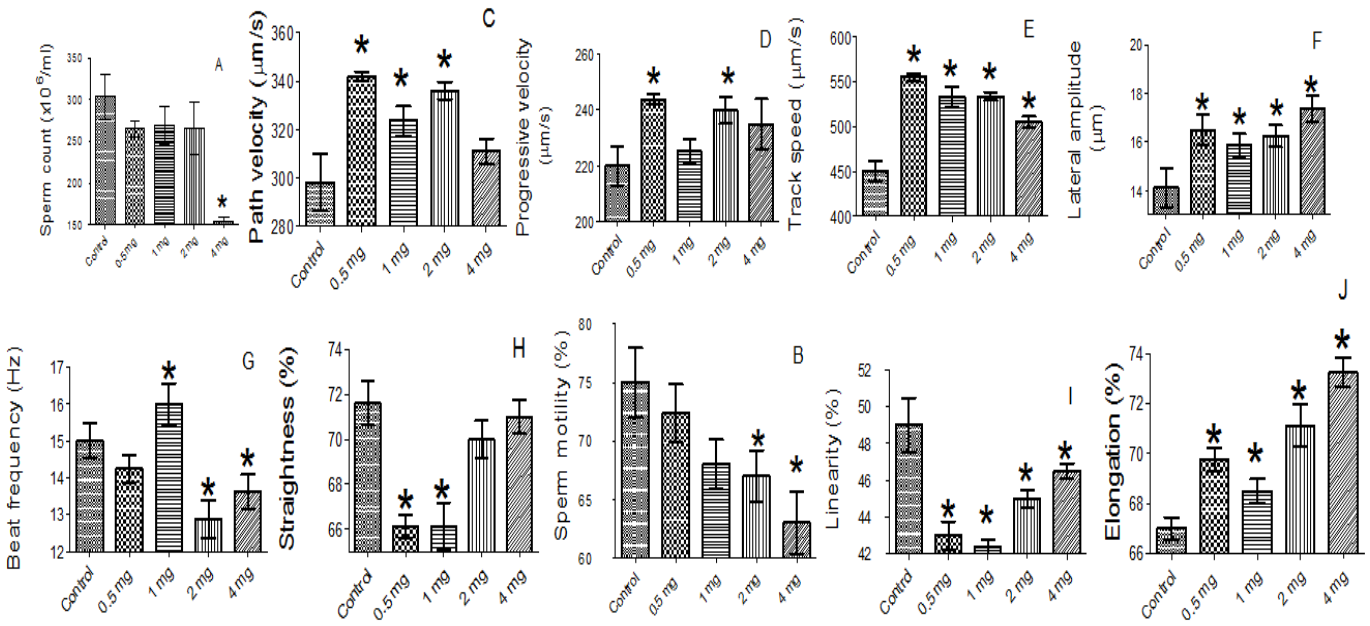

Fig. 2: Sperm count and motility of epididymal samples obtained from control and rats exposed to genistein at oral doses of 0.5 mg, 1 mg, 2 mg and 4 mg /kg body weight for 60 days. Panel A: sperm count results, panel B: sperm motility score, panel C; sperm path velocity, panel D: sperm progressive velocity results, panel E: sperm track speed, panel F: sperm lateral amplitude, panel G: sperm cells beat frequency, panel H: percentage straightness of sperm cells, panel I: percentage linearity assessment of sperm cells, panel J: percentage sperm cells elongation as assessed by CASA.

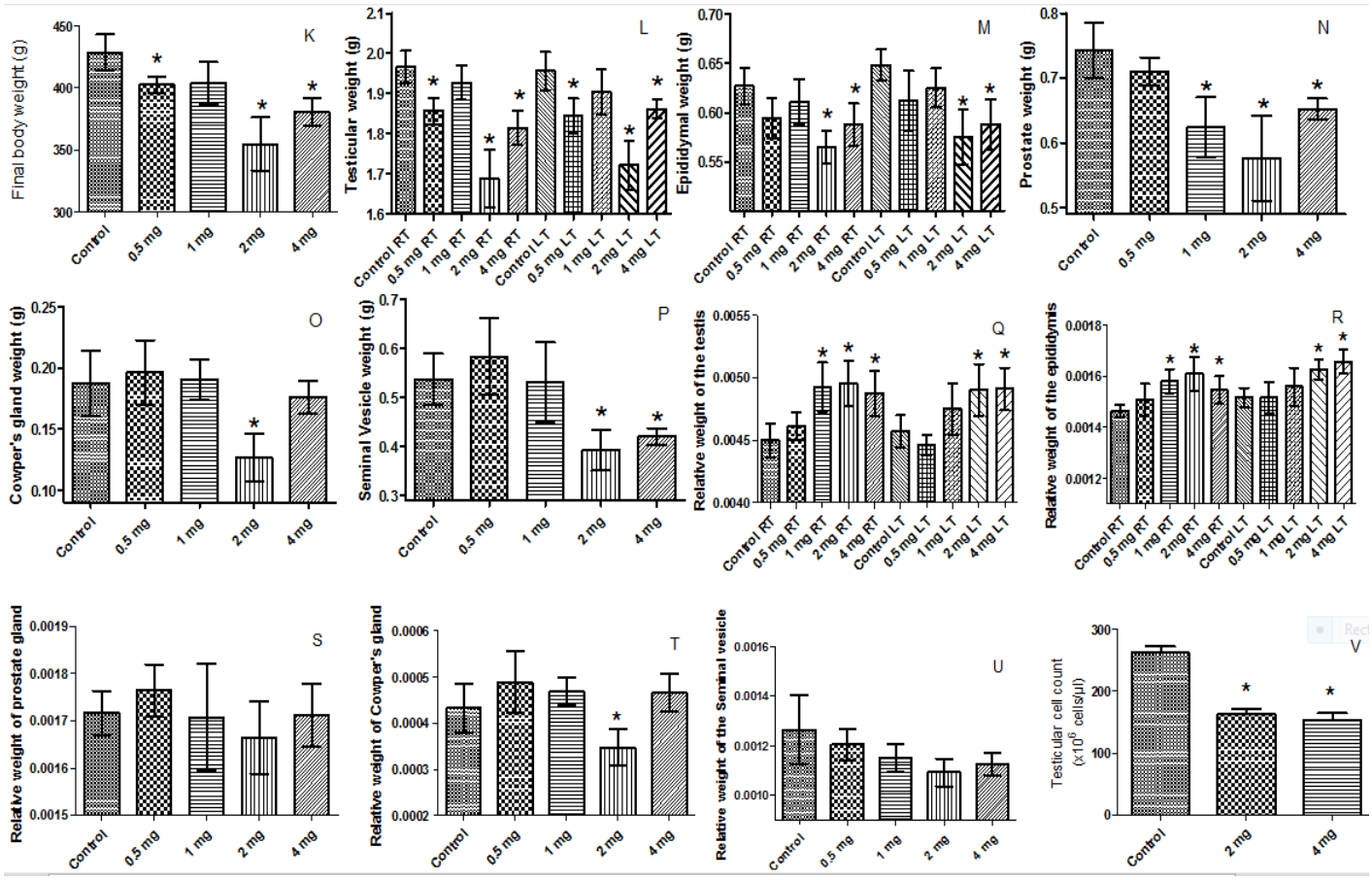

Fig.3: Mean relative weight of some male reproductive organs from control and genistein exposed rats. Panel k: relative testicular weight, panel L: testicular cell count, panel M: relative epididymal weight, panel N: relative prostate gland weight (RT = right, LT =left). Columns represent mean ± SEM; n= 5; *p≤0.05.

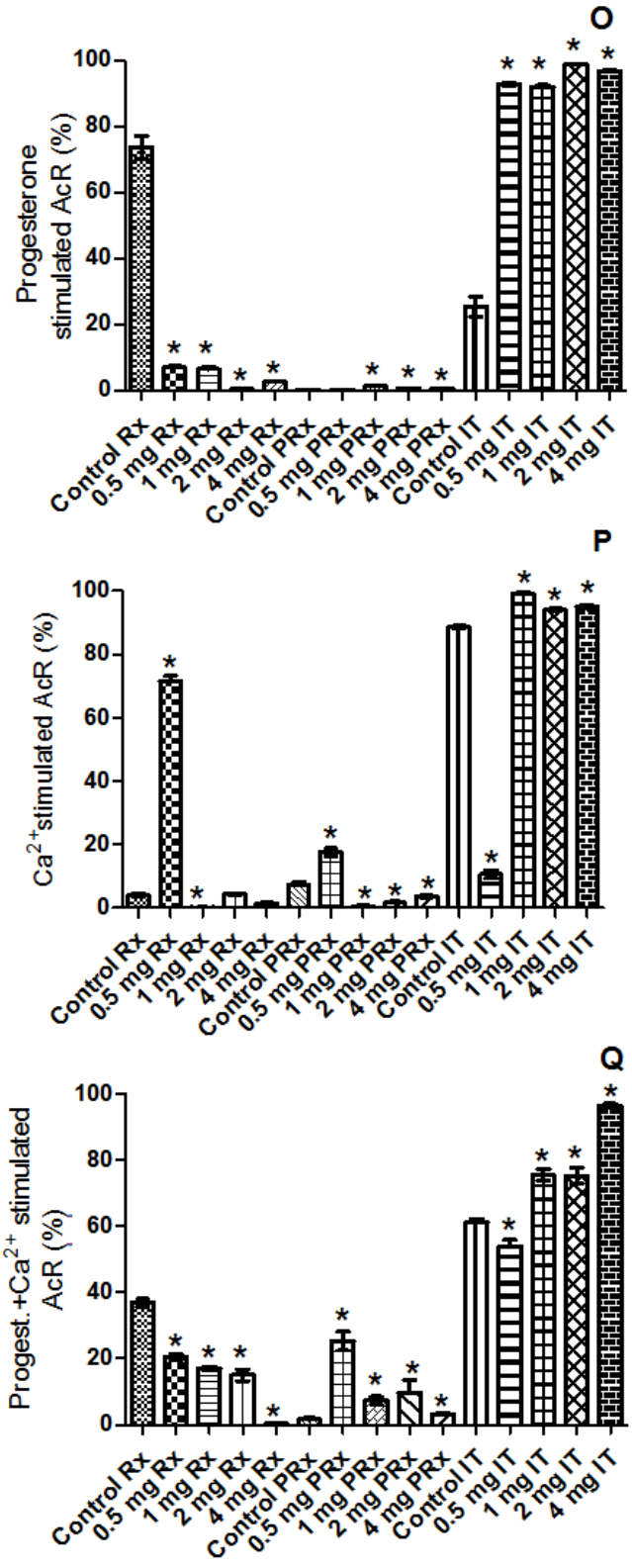

Fig. 4: Acrosome reaction assessment of epididymal sperm cells from control and rats exposed to genistein. Plate O; progesterone stimulated, Plate P; calcium stimulated and Plate Q; progesterone and calcium stimulated acrosome reaction. Rx; reacted, PR; partial reaction and IT; intact acrosome. Columns represent mean ± SEM; n=5; *p ≤ 0.05.

gland was recorded only in the 2 mg/kg genistein group compared with control. The absolute weight of the seminal vesicle was significantly reduced in 2 mg and 4 mg/kg genistein group (Figure 2).

Sperm concentration and motility analysis from epididymis sperm samples obtained from control and rats exposed orally to genistein at a dose of 0.5 mg, 1 mg, 2 mg and 4 mg /kg body weight

The sperm count was significantly reduced in the 4 mg/kg body weight genistein treatment when compared with the control. The sperm path velocity was significantly increased at doses of 0.5 mg/kg, 1 mg/kg and 2 mg/kg when compared with control. The progressive velocity was significantly increased at doses of 0.5 mg/kg and 2 mg/kg. The sperm track speed and lateral amplitude were significantly increased in all treatment groups when compared with the control. Sperm tail beat frequency was significantly increased in the 1 mg/kg group when compared with the control while it was significantly reduced in the 2 mg/kg and 4 mg/kg groups. Sperm straightness was significantly reduced in 0.5 mg/kg and 1 mg/kg group. Sperm motion linearity was significantly decreased in all genistein treated groups when compared with the control. Sperm elongation was significantly increased at the 0.5 mg/kg, 2 mg/kg and 4 mg/kg doses when compared with the control (Fig 1).

Potency (mating index), fertility index and number of days of cohabitation with female rats before successful mating

The duration of days of cohabitation before a successful mating was achieved was significantly increased in the 2 mg/kg and 4 mg/g groups. The potency (mating index) for 2 mg and 4 mg groups were significantly reduced when compared with control. Fertility index was significantly reduced in the 2 mg/kg and 4 mg/kg groups (Fig 4).

Serum testosterone, estradiol and Leptin levels in rats exposed to genistein

Serum testosterone was only significantly reduced in the 0.5 mg/kg group while it was significantly increased in the 1 mg/kg. Serum estradiol level was significantly reduced in all the exposed groups compared to level in the control animals while serum leptin level was significantly increased in the 1 mg/kg group only.

Acrosome reaction assessment of epididymal sperm cells

There was a significant reduction in the percentage of acrosome reacted sperm cells stimulated by progesterone in all the genistein treated rats compared

to control (Fig 3). This was with a concomitant significant increase in the percentage of sperm cells that are intact with no acrosome reaction in all the genistein exposed rats. There was however, a significant increase in the percentage of sperm cells that have undergone partial acrosome reaction in the 1 mg/kg, 2 mg/kg and 4 mg/kg groups stimulated by progesterone. In the calcium induced acrosome reaction, a significant increase and decrease in percentage of AcR were recorded in 0.5 mg/kg and 1 mg/kg group respectively. There was a significant increase in the percentage of acrosome that were intact in the 1 mg/kg, 2 mg/kg and 4 mg/kg groups that were stimulated with calcium. A significant increase in partially reacted acrosomes was recorded in 0.5 mg/kg group while the 1 mg/kg, 2 mg/kg and 4 mg/kg groups recorded significant decrease in the percentage of acrosomes that have undergone partial AcR. Combined stimulation of acrosome reaction with progesterone and calcium significantly increased the percentage of acrosomes that were intact in 1 mg/kg, 2 mg/kg and 4 mg/kg groups, while it was decreased in the 0.5 mg/kg group. The percentage of acrosomes that reacted to the combined stimulation with progesterone and calcium were significantly reduced, while the acrosomes that partially reacted were significantly increased in all the genistein treated groups compared to control (Fig. 3).

Ploidy analysis of testicular tissue from rats orally treated with genistein

The highest dose of genistein decreased the number of haploid (1C), diploid (2C), and S phase cells while the HC2:1C was increased (Table 2). The elongated spermatid HC1 was significantly higher in the 2mg/kg group compared with the control. The total number of round spermatozoa (haploid) was significantly lower in 4 mg/ kg treated group making the total number of haploid cells significantly reduced in the 4 mg/kg treated group compared to control. The 2C non-haploid stage (which contains Sertoli, Leydig, myoid and other interstitial cells), the S-phase (pre-leptotene spermatocyte), the 4C (primary spermatozoa) and the total number of non-haploid cells were significantly reduced in the 4mg/kg genistein treated group compared with the control.

DISCUSSION

Genistein is a phytoestrogen that competes with endogenous estrogen for the beta estrogen receptor (β-ER) binding site (Montani *et al*., 2008). Its influence on male reproductive function in adult *Holtzman* rats was investigated at doses ranging from 0.5 mg/kg to 4 mg/kg body weight and for an exposure duration of 60 days. The results of the feed consumption showed a significant decrease in feed consumption until around the 15th day of the experiment in the genistein treated groups compared with the control, corroborating previous report that genistein decreased feed intake and body weight in rats (Kim *et al*., 2006). The final body weight of rats treated with genistein at doses of 0.5 mg, 2 mg and 4 mg/kg body weight were significantly reduced showing that genistein adversely influenced body weight.

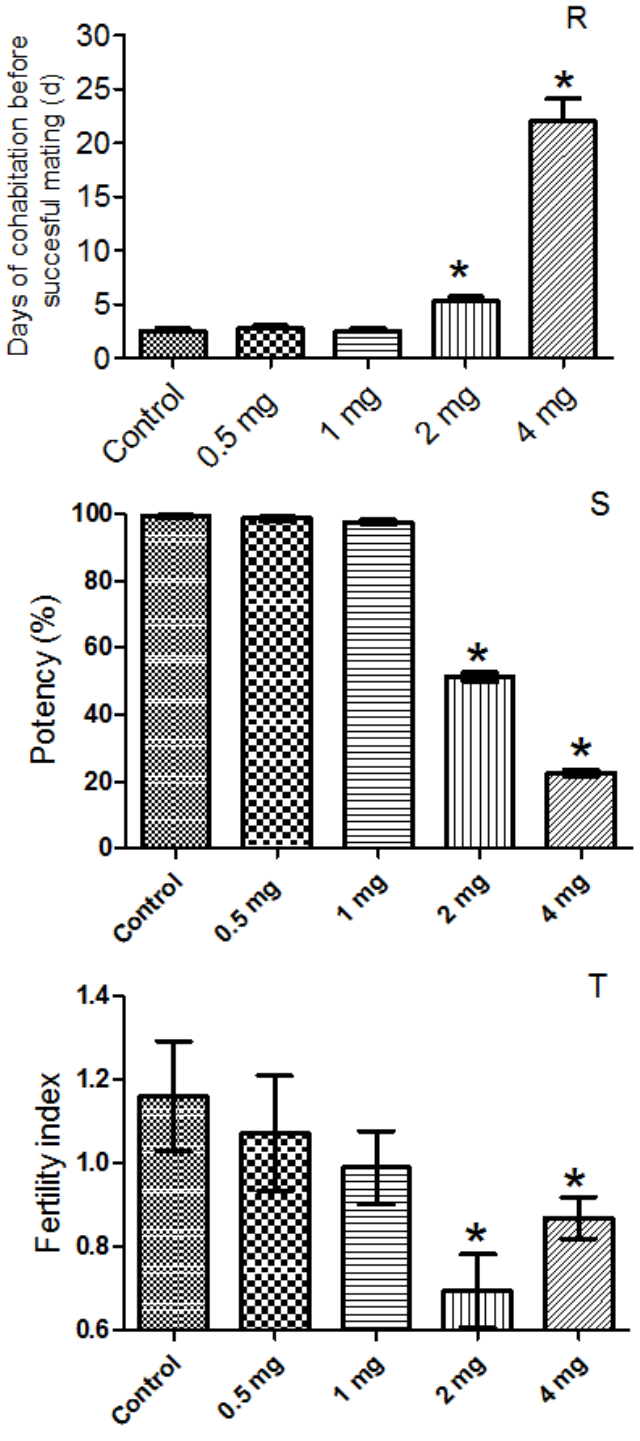

Figure 5: Mating index, Fertility index and number of days of cohabiting with female rats before a successful mating. Columns represent mean ± SEM; n= 5; *p≤0.05.

Table 1: Serum testosterone, estradiol and Leptin levels in rats treated with genistein

Group	Serum leptin (ng/ml)	Serum estradiol (pg/ml)	Serum testosterone (ng/ml)
Control	0.66 ± 0.01	88.33 ± 05.43	2.24 ± 0.10
0.5 mg/kg	0.68 ± 0.01	51.77 ± 6.31*	1.20 ± 0.26*
1.0 mg/kg	0.70 ± 0.02*	45.17 ± 12.42*	6.41 ± 0.52*
2.0 mg/kg	0.66 ± 0.02	21.01 ± 0.24*	2.40 ± 1.94
4.0 mg/kg	NA	NA	NA

NA; sample not available, *Significant different from control of same days of exposure. All values are mean ± S.E.M, n= 5; * $p \leq 0.05$

We have previously reported that genistein adversely interferes with energy homeostasis by influencing leptin synthesis and thus weight gain in laboratory rats (Awobajo *et al*., 2013; 2015) in addition to the report that genistein reduced feed intake (Kim *et al*., 2006) which was also observed in the genistein treated rats in this study. The results of the genistein administration on reproductive organ weight showed significant decrease in both right and left testicular weight in the 0.5 mg/kg, 2 mg/kg and 4 mg/kg genistein groups in spite of the significant increase recorded in the relative weight of the testes. The disparities between the absolute and relative testicular weights were as a result of the significant decrease in the final body weights in the gravid rats that were treated with genistein compared with the control. The significant decrease in the absolute weights of the testes recorded in 2 mg and 4 mg groups were accompanied by significant decrease in testicular cell count (Figure 3, panel- V). Testicular cells comprises of all the cells that are present in the testis and this includes the sperm cells at their various stages of development, sertoli cells, leydig cells, peritubular myoid cells and other interstitial cells (Meistrich *et al*., 1973; Bryant *et al*., 2013).Testicular weight is an indicator of cellular mass and the significant decrease in testicular weights recorded in the genistein treated (0.5, 2 and 4 mg/kg) groups is an indicator of interference with testicular cell growth and spermatogenesis.

Furthermore, the significant decrease recorded in the absolute weight of the right and left epididymis in the 2 mg and 4 mg/kg groups can be an indication of the adverse effects of genistein on the proliferation of the cells of the testis including sperm cells. The epididymal sperm concentration was thus, significantly reduced in the 2 mg and 4 mg/kg group as well as the 0.5 mg/kg group that also recorded a significant decrease in its testicular weight. The epididymis is made up of tortuously coiled thin tubules within which the non-motile sperm cells are stored before their ultimate release at ejaculation (Yeung *et al*., 1991). The epididymal volume is therefore, a reflection of the concentration of the sperm storage with possibilities of a reduction by any factor that can adversely influence spermatogenesis (Awobajo *et al*., 2005; Sharpe 2010). The prostate absolute weight was also significantly reduced in 1 and 2 and 4 mg/kg groups in this study. There are reports of anti-prostate cancer activities of genistein with down regulation of several cell cycle genes including mitotic-kinesins and cyclin dependent kinases (Zhang *et al*., 2008; Merchant *et al*., 2011). Thus genistein may have inhibited the growth of prostate cell via this already established mechanism. A significant decrease in the seminal vesicle weight was also recorded in the 2 mg and 4 mg groups along with a significant decrease in the Cowper's gland weight in 2 mg/kg group. Although, the prostate and the seminal vesicles are not involved in spermatogenesis, they however contribute to the final semen fluid that stabilises sperm chromatin, contributes to sperm flagella motility and suppression of the immune activity in the female reproductive tract. (Gonzales 2001; Kawanoa *et al*., 2014). In this study, there was a significant decrease in both the flagella beat frequency and spermatozoa motility in the 2 mg/kg and 4 mg/kg groups despite the significant increases recorded in the track speed, lateral amplitude, linearity and sperm elongation in the same genistein treated groups.

There are conflicting reports in literature on the possible influence of genistein on reproductive organ weights. There were reports of alteration in reproductive organ weights in humans, rats, mice and in Japanese Quail (Pelletier *et al*., 2000; Delclos et.al., 2001; Lee at al., 2004; Bajpai *et al*., 2003; Wilhelms *et al*., 2006) while others reported no effect on reproductive organ weights in rat and mice (Fritz *et al*., 2003; Jung *et al*., 2004; Lee *et al*., 2004; Montani *et al*., 2008; Intarapat *et al*., 2014; Intarapat *et al*., 2016). This study however, confirmed that oral administration of genistein, precisely at 2 and 4 mg/kg altered some male reproductive organ weights and possibly their functions.

Genistein exerted a modulating effect on testosterone production, with a significant decrease in 0.5 mg/kg group and an increase in 1 mg/kg group (Table 1). The decrease recorded corroborates the report of Weber *et al*. (2001). In their study, exposure of male rats to low

Table 2: Ploidy analysis of testicular tissue for spermatogenesis in male exposed to genistein

Group [α]	Total GC recovered /10000 cells	Germ cell populations (%)[δ]									
		Haploids						Non-haploid			
		HC2	HC1	1C	Total	HC2/HC1	HC2:1C	2C	S-phase	4C	Total
Control	70.68 ± 5.85	29.82 ± 5.85	10.56 ± 1.15	20.14 ± 4.71	60.52 ± 1.49	2.80 ± 0.39	1.99 ± 0.61	4.82 ± 0.39	1.06 ± 0.61	1.66 ± 0.71	7.54 ± 0.15
2 mg Gen	69.78 ± 3.67	21.18 ± 8.59	19.08 ± 2.46*	22.68 ± 4.28	62.95 ± 3.90	1.62 ± 0.83	1.72 ± 0.95	4.82 ± 1.20	0.57 ± 0.15	1.27 ± 0.50	6.65 ± 1.18
4mg Gen	55.60 ± 4.58†	33.47 ± 2.53	10.27 ± 3.18	9.20 ± 1.48†	52.93 ± 4.34†	3.35 ± 0.58	3.71 ± 0.30*	1.97 ± 0.40†	0.23 ± 0.13†	0.40 ± 0.32†	2.60 ± 0.40†

[α]n = 5 rats/group; † Significant reduction $p \leq 0.05$, 2C-Spermatogonia (will also include Sertoli, Leydig, myoid and other interstitial cells as they are also diploid (2n); S-phase - Pre-leptotene spermatocytes; 4C- Primary spermatozoa (Nonhaploid) IC-Round spermatozoa (Haploid); HCI-Elongating spermatid (Haploid); HC2-Elongated spermatids (Haploid); [δ] Germ cells populations were calculated considering the total % recovery values as 100%. Values are mean ± SEM

dose of genistein was found to down regulate testosterone production by decreasing steroidogenic response of Leydig cells via both ERα and ERβ. Genistein has also been reported to down-regulate the expression of mitochondrial P450 side-chain cleavage enzyme, which catalyses the conversion of cholesterol to pregnenolone; an important step in steroidogenesis and synthesis of testosterone (Svechnikov *et al*., 2005). On the contrary, genistein has been reported to promote testosterone production from isolated Leydig cells *in vitro* at very low concentrations while at the same time inhibiting testosterone synthesis at higher concentrations (Zhu *et al*., 2009). The estradiol assay results showed a dose dependent decrease in serum estradiol with increasing concentration of genistein administered. This result corroborates the report of Kao *et al*., (1998) and Shanle and Xu, (2011) where they reported that genistein like any other phytoetsrogen can modulate metabolic enzymes that are crucial for normal oestrogen synthesis and metabolism leading to lower endogenous oestrogen levels. Although, phytoestrogen or any other endocrine disrupting chemical (EDC) usually operates via multiple mechanisms, a few of such mechanisms that have been identified include; aryl hydrocarbon receptor (AhR) and cytochrome P450 aromatase (Shanle and Xu, 2011). Genistein has been well shown to have general estrogenic influences on most of the body systems but its effects have been reported to be majorly modulated via the beta oestrogen receptors (ERβ) which are spatially localized and expressed at various levels in the male rat reproductive organs (Pelletier *et al*., 2000; Boukari *et al*.,2003).

Although, serum leptin levels remained unaffected in most of the genistein treated groups in this study, it was however significantly increased in the 1 mg/kg group. Leptin is an adipocyte-derived hormone which has an important role in body weight homeostasis and energy balance. It has recently been shown to play a role as a neuroendocrine mediator in different systems, including the reproductive system (Lapiao *et al*., 2009). Low concentration of leptin or leptin receptor deficiency has also been shown to decrease gonadotrophins secretions (FSH and LH), which will precipitate delayed puberty, increased germ cell apoptosis and impaired spermatogenesis. (Carro *et al*., 1997; Strobel *et al*., 1998; Bhat *et al*., 2006). The present study thus, suggests that genistein might not influence the various male reproductive functions assessed via the leptin mechanism. Other authors have reported that exposing male rats at the stage of pubertal development to genistein did not significantly affect male reproductive functions like sperm indices and reproductive organ weights (Lee *et al*., 2004). This was contrary to the findings in this study.

When the testicular tissue samples were further subjected to ploidy analysis (Table 2), there was a significant reduction in the population of total cells recovered from the testicular tissue and in the population of the non-haploid cells in the 4 mg genistein group. The non-haploid cells include the primary spermatozoa (4C), pre-leptotene spermatocytes (S-phase) and the spermatogonia (2C) cells. A significant reduction in the population of the haploid germ cells was also recorded at the round spermatozoa (IC) stage of spermatogenesis. Spermatogenesis is the process via which the male germ cells are produced in the testes and the process, which is testosterone dependent, is known to be compromised by environmental assaults which include changes to the chemical composition, pH and temperature of the fluid milieu around the testes. Thus, the spermatogenesis arrest and marked reduction in prostrate and seminal vesicle absolute weights recorded in the male rats exposed to genistein in 2 mg/kg and 4 mg/kg and/or 1 mg/kg group will contribute to the adverse effects of genistein on male reproduction in laboratory rats.

Genistein is a phytoestrogen with greater affinity for ERβ, and it has a wide array of effects on the body including the reproductive system. Some of the well documented effects of genistein include its role as α-glucosidase inhibitor (major factor in the sustenance of sperm motility) (Lee and Lee, 2001), osteoblast up regulator and osteoclast down regulator in bone metabolism (Bitto *et al*., 2008), DNa-topoisomerase-II inhibitor (topoisomerase-II mediates DNA modifications and chromatin changes during maturation of spermatogenic cells (Constantinou *et al*., 1995; Chen and Longo 1996; Sukhacheva *et al*., 2003;) to mention but a few.

Further analysis of the motility of the sperm cells showed a significant decrease in the sperm motility and beat frequency in rats exposed to 2 mg and 4 mg genistein. Sperm motility is a combination of several factors among which are path velocity, progressive velocity, tract speed, lateral amplitude and beat frequency (Hirano *et al*., 2001; Rhemrev *et al*., 2001). The beat frequency is an indicator of flagella swimming ability (Hong *et al*., 1993) and a direct function of the mitochondria found in abundance at the flagella of the sperm cell serving as the site where ATP is generated for motility. Svechnikov *et al*. (2005) has reported the cytochrome P450 inhibitory activities of genistein which may partly explain its adverse effect on sperm motility as recorded in this experiment. The importance of tyrosine phosphorylation in sperm capacitation and ultimately the regulation of sperm motility has been well documented (Bajpai *et al*., 2003). Genistein activity as a protein kinase inhibitor (Akinyama *et al*., 1987) will truncate this important biochemical process with adverse consequences on sperm motility (Mahony and Gwathmey 1999). Genistein has also been reported to inhibit α-glucosidase an important factor in the sustenance of sperm motility (Lee and Lee, 2001). Thus, adverse influence of these magnitudes on sperm function as precipitated by genistein can be considered enough evidence to potentiate infertility (Kato *et al*., 2002; Fukushima *et al*., 2005). Our previous study on the influence of genistein on induction of pregnancy and foetal development showed that female rats that were exposed to genistein experienced disruption and delay in fertilization of ovulated oocytes (Awobajo *et al*., 2013). These adverse effects were accompanied with an increased pre-and post-implantation loss as well as decreased placental and foetal weights at different gestational days and at term (Awobajo *et al*., 2013). We also reported that these adverse effects were predicated on genistein activities in modifying thyroid hormone, C-reactive protein and leptin synthesis during pregnancy (Awobajo *et al*., 2015). Apart from the sperm concentration, sperm motility and morphology, another important consideration in assessment of male fertility is the determination of the ability of live spermatozoa to fertilize the oocyte at the point of contact. This process requires a procedure known as acrosome reaction. In this study, genistein suppressed acrosome reaction (AcR) in all the genistein treated groups when AcR was stimulated with progesterone (Figure 4). However, genistein suppression of AcR was significantly reduced in progesterone+Ca^{+} stimulated AcR. In this study, progesterone initiated AcR while Ca^{+} alone failed to activate AcR. Previous reports showed the importance of 12-O-tetradecanoylphorbol 13-acetate (a protein kinase-C activator) in the activation of human sperm AcR and capacitation suggesting the involvement of a protein kinase C in the pathway of sperm AcR (Furuya *et al*., 1993). Genistein as a tyrosine kinase inhibitor (Akinyama *et al*., 1987) has been shown to inhibit the induction of acrosome reaction in cat and mouse (Furuya *et al*., 1992; Yanagimachi, 1994; Pukazhenthi *et al*., 1998) thus preventing sperm hyperactivity. In an *in vivo* study, it was reported that genistein inhibited tyrosine phosphorylation of sperm tail protein and blocked capacitation and subsequent sperm hyperactivity (Mahony and Gwathmey, 1999). This *in vivo* effect may be associated with a decrease in the fertilizing ability of the sperm cells.

In conclusion exposure of male *Holtzman* rats to genistein inhibited testicular cell growth and differentiation with resultant adverse effect on sperm motility.

ACKNOWLEDGEMENT

This work was sponsored by The World Academy of Sciences and the Department of Biotechnology India (TWAS-DBT India) during Dr. Awobajo's Postdoctoral fellowship visit to National Institute for Research in Reproductive Health India. The authors express deep appreciation for the assistance rendered by Mr. Suryakant Mandavkar in animal experimentation and tissue processing. We also express our gratitude to members of staff of the Animal House and Neuroendocrinology Department, NIRRH, Mumbai, India for their assistance at different stages of the research. This research work was jointly funded by The World Academy of Sciences and the Department of Biotechnology India.

Declaration of conflict of interest: Authors declare that there is no conflict of interest in this study.

REFERENCES

Akinyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., Fukami, Y. (1987). Genistein, a specific inhibitor of tyrosine-

specific protein kinases. J. Biol. Chem. 262(12):5592-5595.

Awobajo, F. O., Uzor, P.O.**,** Onokpite, B.O., Yekinni, M.A., Babaleye, T.A., Tijani. K.O. (2015). Genistein precipitated hypothyroidism, and altered leptin and C-reactive protein synthesis in pregnant rats. Nig. J. Physiol. Sci. 30:79-85.

Awobajo, F.O., Nandedkar, T.D., Balasinor, N.H. (2013). Genistein alters oestrous cyclicity, oocyte fertilization and implantation process in rats. Nig. Qt. J. Hosp. Med. 23(3):188- 193.

Badkoobeh, P., Parivar, K., Kalantar, S.M., Hosseini, S.D., Salabar, A. (2013). Effect of nano-zinc oxide on doxorubicin- induced oxidative stress and sperm disorders in adult male Wistar rats. Iran J. Reprod. Med.11(9):355-364.

Bajpai, M., Asin, S., Doncel, G.F. (2003). Effect of tyrosine kinase inhibitors on tyrosine phosphorylation and motility parameters in human sperm. Arch. Androl. 49(3):29-46.

Bhat, G.K., Sea, T.L., Olatinwo, M.O., Simorangkir, D., Ford, G.D., Mann, D.R. (2006). Influence of leptin deficiency on testicular morphology germ cell apoptosis and expression evel of apoptosis related genes in the mouse. J. Androl. 27:302-310.

Bitto, A., Burnett, B.P, Polito, F., Marini, H., Levy, R.M., Armbruster, M.A., Minutoli, L., Di Stefano, V., Irrera, N., Antoci, S., Granese, R., Squadrito, F., Altavilla, D. (2008). Effects of genistein aglycone in osteoporotic, ovariectomized rats: a comparison with alendronate, raloxifene and oestradiol. Br. J. Pharmacol. 155(6):896–905.

Boukari K, Ciampi ML, Guiochon-Mantel A, Young J, Lombès M, Meduri G. (2007). Human fetal testis: source of estrogen and target of estrogen action. Hum. Reprod. 22:1885–1892.

Bouker, K.B., Hilakivi-Clarke, L. (2000). Genistein: does it prevent or promote cancer? Environ. Healt. Perspect. 108:701-708.

Breinholt, V., Hossaini, A., Svendsen, G.W., Brouwer, C., Nielsen, E. (2000). Estrogenic activity of flavonoids in mice, the importance of estrogen receptor distribution, metabolism and biovailability. Food Chem. Toxicol. 38:555–564.

Bryant, J.M., Meyer-Ficca, M.L., Dang, V.M., Berger, S.L., Meyer, R.G. (2013). Separation of Spermatogenic Cell Types Using STA-PUT Velocity Sedimentation. *J. Vis. Exp.* (80), e50648, doi:10.3791/50648.

Calogero, A.E., Burrello, N., Barone, N., Palermo, I., Grasso, U., D'Agata, R. (2000). Effects of progesterone on sperm function: mechanisms of action. Human Reprod. 15(1):28-45.

Carro, E., Pinilla, L., Scoane, L.M.,1997. Influence of endogenous leptin tone on the estrous cycle and luteinizing hormone pulsatility in female rat. Neuroendo. 66:375-377.

Chen J.L., Longo F.J. (1996). Expression and localization of DNA topoisomerase II during rat spermatogenesis. Mol. Reprd. Dev. 45(1):61-71.

Close B.,Banister K., Baumans V., Bernoth E.M., Bromage N., Bunyan J., Erhardt W., Flecknell P., Gregory N., Hackbarth H., Morton D., Warwick C. (1996). Recommendations for euthanasia of experimental animals: Part 1. LaboratoryAnimals 30:293-316.

Constantinou, A., Mehta, R., Runyan, C., Rao, K., Vaughan, A., Moon, R. (1995). Flavonoids as DNA topoisomerase antagonists and poisons: structure-activity relationships. J. Nat.Prod. 58(2):217-225.

Cooke, P.S., Nanjappa, M.K., Ko, C., Prins, G.S., Hess, R.A. (2017). Estrogens in male physiology. Physiological Reviews. 97(3): 995-1043.

Delclos, K.B., Bucci, T.J., Lomax, L.G., Latendresse, J.R., Warbritton, A., Weis, C.C., Newbold, R.R. (2001). Effects of dietary genistein exposure during development on male and female CD (Sprague-Dawley) rats. Reprod. Toxicol. 15(6):647-663.

Dumasia, K., Kumar, A., Kadam, L., Balasinor, N.H. (2015). Effect of estrogen receptor-subtype specific ligands on fertility in adult male rats. J. Endocrinol. 225:169-180.

Faqi, A.S., Johnson, W.D., Morrissey, R.L., McCormick, D.L. (2004). Reproductive toxicity assessment of chronic dietary exposure to soy isoflavones in male rats. Reprod. Toxicol. 18:605-611.

Fritz, W.A., Cotroneo, M.S., Wang, J., Eltoum, I.E., Lamartiniere, C.A. (2003). Dietary diethylstilbestrol but not genistein adversely affects rat testicular development. J. Nutr. 133(7):2287-93.

Fukushima, T., Kato, M., Adachi, T., Hamada, Y., Horimoto, M., Komiyama, M., Mori, C., Horii, I. (2005). Effects of Sulfasalazine on Sperm Acrosome Reaction and Gene Expression in the Male Reproductive Organs of Rats. Toxicol. Sci. 85:675-682.

Furuya, S., Endo, Y., Oba, M., Nozawa, S., Suzuki, S. (1992). Effects of modulators of protein kinases and phosphatases on mouse sperm capacitation. J. Assist. Reprod. Genet. 9:391-399.

Furuya, S., Endo, Y., Oba, M., Suzuki, S., Nozawa, S. (1993). Effects of epidermal growth factor on human sperm capacitation. Fertil. Steril. 60:905-910.

Gill-Sharma M.K., Balasinor N., Parte P. (2001). Effect of intermittent treatment with tamoxifen on reproduction in male rats. Asian J Androl. 3(2):115-119.

Gonzales G.F. (2001). Function of seminal vesicles and their role on male fertility. Asian J Androl. 3:251-258.

Gupta, A.K., Bhandari, B.,2011. Acrosome reaction; relevance of zona pellucida glycoprotein. Asian J. Androl. 13(1):97-105.

Hirano, Y., Shibahara, H., Obara, H., Suzuki, T., Takamizawa, S., Yamaguchi, Y., Tsunoda, H., Sato, I. (2001). Relationships between sperm motility characteristics assessed by the computer-aided sperm analysis (CASA) and fertilization rates *in vitro*. J. Assist. Reprod. Genet. 18(4):213-218.

Hong, C.Y., Lee, M.F., Ou, M.C., Chao, H.T. (1993). Tail beat frequency of human sperm: evaluated with sperm head fixation method and computer-assisted semen analysis. Arch. Androl. 30(3):171-176.

Hsieh, C.Y., Santell, R.C., Haslam, S.Z., Helferich, W.G. (1998). Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells in vitro and in vivo. Cancer Res. 158(17):3833-3838.

Intarapat, S., Achariya, S., Orawan, S. (2014). Anatomical and Histological Changes of Reproductive Organs in Japanese Quail (Coturnix japonica) Embryos after in ovo Exposure to Genistein. Int. J. Poult. Sci. 13:1-13.

Intarapat, S., Sailasuta, A., Satayala, O. (2016). Genistein causes germ cell reduction in the genital ridges of Japanese quail Coturnix japonica embryo. Int. J. Poult. Sci.13 (1):1-13.

Jefferson, W.N., Patisaul, H.B., Williams, C.J. (2012). Reproductive Consequences of Developmental Phytoestrogen Exposure. Reprod. 143(3):247-260.

Jung, E.Y., Lee, B.J., Yun, Y.W., Kang, J.K., Baek, I.J., Jurg, M.Y. (2004). Effects of exposure to genistein and estradiol on reproductive development in immature male mice weaned from dams adapted to a soy-based commercial diet. J. Vet. Med. Sci. 66:1347-1354.

Jung, E.Y., Lee, B.J., Yun, Y.W., Kang, J.K., Baek, I.J., Yon, J.M., Lee, Y.B., Sohn, H.S., Lee, J.Y., Kim, K.S., Yu, W.J., Do, J.C., Kim, Y.C., Nam, S.Y. (2004). Effects of exposure to genistein and estradiol on reproductive development in immature male mice weaned from Das adapted to a soy-based commercial diet. J. Vet. Med. Sci. 66(11):1347-1354.

Kao, Y.C., Zhou, C., Sherman, M., Laughton, C.A., Chen, S. (1998). Molecular basis of the inhibition of human aromatase (estrogen synthetase) by flavone and isoflavone phytoestrogens: A site-directed mutagenesis study. Environ. Healt. Perspect. 106:85-92.

Kato, M., Makino, S., Kimura, H., Ota, T., Furuhashi, T., Nagamura, Y.,Hirano, K. (2002). In vitro evaluation of acrosomal status and motility in rat epididymal spermatozoa treated with a-chlorohydrin for predicting their fertilizing capacity. J. Reprod. Dev. 48: 461-468.

Kawanoa N., Arakib N., Yoshidac K., Hibinod T., Ohnamia N., Makinoa M., Kanaia S., Hasuwae H., Yoshidab M., Miyadoa K., Umezawaa A. (2014). Seminal vesicle protein SVS2 is required for sperm survival in the uterus. Developmental Biology. 111(11):4145–4150.

Kim H.E., Nelson-Dooley C., Della-Fera M.A., Yang J.Y., Zhang W., Duan J., Hartzell D.L., Hamrick M.W., Baile C.A. (2006). Genistein decreases food intake, body weight, and fat pad weight and causes adipose tissue apoptosis in ovariectomized female mice. J. Nutr. 136(2): 409-414.

Landauer, M.R., Srinivasan, V., Seed, T.M. (2003). Genistein treatment protects mice from ionizing radiation injury. J Appl. Toxicol. 23:379–385.

Lapiao, F., Agarwal, A., du Plessis, S.S. (2009). The role of insulin and leptin in male reproduction. Arch. Med. Sci. 5(1A): S48–S54.

Lee, B., Jung, E., Yun, Y., Kang, J., Baek, I., Yon, J., Lee, Y., Sohn, H., Lee, J., Kim, K., Nam, S.,2004. Effects of exposure to genistein during pubertal development on the reproductive system of male mice. J. Reprod. Dev. 50(4):399-409.

Lee, B.J., Kang, J.K., Jung, E.Y., Yun, Y.W., Baek, I.J., Yon, J.M., Lee, Y.B., Sohn, H.S., Lee, J.Y., Kim, K.S., Nam, S.Y. (2004). Exposure to genistein does not adversely affect the reproductive system in adult male mice adapted to a soy-based commercial diet. J Vet Sci. 5(3):227-34.

Lee, D.S., Lee, S.H. (2001). Genistein, a soy isoflavone, is a potent alpha-glucosidase inhibitor. FEBS Lett. 501:84–86.

Leyton, L., LeGuen, P., Bunch, D., Saling, P.M. (1992). Regulation of mouse gamete interaction by sperm tyrosine kinase. Proc. Natl. Acad. Sci. 89:11692-11695.

Lian-Dong, Z., Qian, D., Zi-Ming, W., Ming, G., Lei, W., Tie, C., He-Cheng, L. (2013). Disruption of reproductive development in male rat offspring following gestational and lactational exposure to di-(2-ethylhexyl) phthalate and genistein. Biol Res. 46:139-146.

Mahony, M.C., Gwathmey, T. (1999). Protein tyrosine phosphorylation during hyperactivated motility of phosphorylation during hyperactivated motility of cynomolgus monkey. (Macaca fascicularis) spermatozoa. Biol. Reprod. 60:1239-1243.

Meistrich, M.L., Bruce W.R., Clermont Y. (1973). Cellular composition of fractions of mouse testis cells following velocity sedimentation. Experimental Cell Research. 79(1):213-227.

Merchant, K., Kumi-Diaka J., Rathinavelu A., Esiobu N., Zoeller R., Hartmann J., Johnson M. (2011). Molecular basis of the anti-cancer effects of genistein

isoflavone in LNCaP prostate cancer cells. Functional Foods in Health and Disease. 1(3):91-105.

Montani, C., Penza, M., Jeremic, M., Biasiotto, G., Sala, G.L., Felici, M.D., Ciana, P., Maggi, A., Lorenzo, D.D. (2008). Genistein is an Efficient estrogen in the whole-body throughout Mouse Development. Toxicol Sci. 103 (1):57-67.

Nagao, T., Yoshimura, S., Saito, Y., Nakagomi, M., Usumi, K., Ono, H. (2001). Reproductive effects in male and female rats of neonatal exposure to genistein. Reprod. Toxicol.15:399-411.

National Advisory Committee For Laboratory Animal Research (NACLAR) (2004). Guidelines on the care and use of animals for scientific purposes. 1-149.

Opalka, M., Kaminska, B., Ciereszko, R., Dusza, L. (2004). Genistein affects testosterone secretion by leydig cells in roosters (Gallus gallus domesticus). Reprod. Biol. 4:185-193.

Pelletier, G., Labrie, C., Labrie, F. (2000). Localization of oestrogen receptor alpha, oestrogen receptor beta and androgen receptors in the rat reproductive organs. J. Endocrinol. 165:359-370.

Pukazhenthi, B.S., Wildt, D.E., Ottinger, M.A., Howard, J. (1998). Inhibition of domestic cat spermatozoa acrosome reaction and zona pellucida penetration of tyrosine kinase inhibitors. Mol. Reprod. Dev. 49:48-57.

Rhemrev, J.P.T., Lens, J.W., McDonnell, J., Schoemaker, J., Vermeiden, J.P.W. (2001). The post wash total progressively motile sperm cell count is a reliable predictor of total fertilization failure during *in vitro* fertilization treatment. Fertil. Steril. 76(5):884–891.

Setchell, K.D., Zimmer-Nechemias, L., Cai, J., Heubi, J.E. (1998). Isoflavone content of infant formulas and the metabolic fate of these phytoestrogens in early life. Am. J. Clin. Nutr. 8,1453S–1461S.

Shanle, E.K., Xu, W. (2011). Endocrine disrupting chemicals targeting estrogen receptor signaling: identification and mechanisms of action. Chem. Res. Toxicol. 24:6–19.

Sharpe, R.M. (2010). Environmental/lifestyle effects on spermatogenesis. Philos Trans R Soc. LondBBiol.Sci. 365(1546):1697-1712.

Strobel, A., Issad, T., Camoin, L., Ozala, M., Strosberg, D. (1998). A leptin missense mutation associated with hypogonadism and morbid obesity. Nat. Gent. 18:213-215.

Sukhacheva T. V., Bogush T. A., Kolomiets O. L. (2003).Destructive Effect of DNA Topoisomerase II Inhibitor Vepesid on Mouse Spermatogenesis. Bulletin of Experimental Biology and Medicine. 135(5):464-469.

Svechnikov, K., Supornsilchai, V., Strand, M.L., Wahlgren, A., Seidlova-Wuttke, D., Wuttke, W., Soder, O. (2005). Influence of long-term dietary administration of procymidone, a fungicide with anti-androgenic effects, or the phytoestrogen genistein to rats on the pituitary-gonadal axis and Leydig cell steroidogenesis. J. Endocrinol. 187:117-124.

The University of Texas at Austin Institutional Animal Care and Use Committee (2013) Guidelines for the use of cervical dislocation for rodent euthanasia. Guideline # 004:1-2.

Tian, T., Li, J., Li, B., Wang, Y., Li, M., Ma, D., Wang, X. (2014). Genistein exhibits anti-cancer effects via down-regulating FoxM1 in H446 small-cell lung cancer cells. Tumour Biol. 35(5):4137-45.

Weber, K.S., Setchell, K.D.R., Stocco, D.M., Lephart, E.D. (2001). Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate 5α-reductase or testicular steroidogenic acute regulatory peptide levels in adult male Sprague-Dawley rats. J Endocrinol 170:591-599.

Whitten, P.L., Naftolin, F. (1998). Reproductive actions of phytoestrogens. Baillieres. Clin. Endocrinol. Metab. 12(4),667-690.

Wilhelms, K.W., Scanes, C.G., Anderson, L.L. (2006) Lack of Estrogenic or Antiestrogenic Actions of Soy Isoflavones in an Avian Model: The Japanese Quail. Poult Sci. 85 (11):1885-1889.

Wisniewski, A.B., Klein, S.L., Lakshmanan, Y., Gearhart, J.P. (2003). Exposure to genistein during gestation and lactation demasculinizes the reproductive system in rats. J. Urol. 169:1582–1586.

Yanagimachi, R. (1994). Mammalian fertilization. In: Knobil E, Neil (eds). The Physiology of Reproduction. New York: Raven Press; 189-317.

Yeung, C.H., Cooper, T.G., Bergmann, M., Schulze, H. (1991). Organization of tubules in the human caput epididymidis and the ultrastructure of their epithelia. Am J Ant. 191(3):261-279.

Zhang L.I., Li L., Wu D.P., Fan J.H., Li X., Wu K.J., Wand Z.Y., He D.L. (2008). A novel anti-cancer effect of genistein: reversal of epithelial mesenchymal transition in prostate cancer cells. Acta Pharmacol Sin. 29 (9):1060-1068.

Zhu, F.R., Wang, Y.G., Chen, J., Hu, Y.X., Han, F.S., Wang, H.Y. (2009). Effects of phytoestrogens on testosterone production of rat Leydig cells. Zhonghu Nan Ke Xue. 15(3):207-11.