Evidence of Regeneration of Testicular and Epididymal Tissue Structure and Function Following Withdrawal from Sub-chronic Khat Exposure: Studies in the Rabbit Animal Model

Albert W. Nyongesa[1*], Esther M. Maluki[2] and Jemimah A. Simbauni[2]

[1]*Department of Veterinary Anatomy and Physiology, University of Nairobi, P.O.Box 30197-00100, Nairobi, Kenya.*
[2]*Department of Zoological Sciences, Kenyatta University, P.O.Box 43844-00100, Nairobi, Kenya.*

Authors' contributions

This work was carried out in collaboration among all authors. Author AWN conceived the idea and designed the experiment, interpreted hormonal and histological data and results reporting and provided critical analysis to paper writing and formatting. Author EMM designed the experiment, contributed reagents and materials, performed the experiments. Author JAS contributed to the experimental design, performed critical analysis of data and paper writing. All authors read and approved the final manuscript.

Article Information

Original Research Article

ABSTRACT

Khat, *Catha edulis*, use is rampant in Eastern Africa and Middle East countries with associated reports of reproductive function impairment in the body of the user. Reports on recovery post long-term khat exposure are obscure. The present study investigated evidence of restoration of testicular and epididymal structure and function during withdrawal from cytotoxic damage caused by sub-chronic exposure of khat extract. Twenty-eight male rabbits were divided into 7 groups of 4 rabbits each. Group I (control) was administered normal saline while groups II, III and IV were administered 1.0 g/kg, 10 g/kg and 20 g/kg body weight of khat extract, respectively, via oral gavage on alternate days of the week for 12 weeks. Blood samples from animals were collected for hormonal assays

Corresponding author: E-mail: albertnyongesa2011@gmail.com;

followed by euthanasia using 26.4 mg/kg body weight of Sagatal sodium intramuscularly for testicular and epididymal histology. Group V, VI and VII were administered 1.0 g/kg, 10 g/kg and 20 g/kg body weight of khat extract, respectively, orally on alternate days of the week for 12 weeks followed by 1-month withdrawal period, blood samples collected for hormone assays and animals sacrificed for testicular and epididymal histology. High khat dose, 20 g/kg body weight, at sub-chronic exposure caused degeneration in spermatogenic cells with accompanying decrease in plasma FSH and testosterone. Histological output of Sertoli cells, Leydig cells and epididymal epithelium appeared unaffected in treatment groups. Post withdrawal data showed apparent regeneration of seminiferous epithelium and restoration of plasma FSH and testosterone comparable to control. It appears khat extract preferentially affected germ cell spermatogonia and subsequent daughter cells while stem cell spermatogonia were unaffected and contributed to regeneration of germinal epithelium and endocrine function.

Keywords: Khat overuse; testicular damage; reproductive hormones; withdrawal; testicular recovery.

1. INTRODUCTION

Spermatogenesis and steroidogenesis are testicular reproductive processes controlled by pituitary gonadotropins: Follicular Stimulating Hormone (FSH) and Luteinizing Hormone (LH). FSH binds to Sertoli cells and spermatogonia [1] within the seminiferous tubules. The binding of FSH to Sertoli cells leads to accumulation of cAMP, activation of protein kinase and subsequent production of androgen binding protein [2]. Dorrington and Armstrong [3] earlier demonstrated that FSH also stimulates conversion of testosterone to βestradiol by Sertoli cells although this role was proven to be insufficient in maintenance of spermatogenesis [4]. Luteinizing hormone (LH), on the other hand, plays a critical role in testicular steroidogenesis [5]. This role is supported by reports in other studies where exposure to environmentally relevant bisphenol A, a monomer in polycarbonate plastics, had adverse effects on pituitary gland LH secretion and impairment of testicular Leydig cell steroidogenesis [6]. Previous studies have shown susceptibility of these gonadotropins to various endocrine disrupters in humans [7]. Habitual khat (*Catha edulis* Forsk), a chewable stimulant of euphoric use, has been implicated as one such endocrine disrupter [8].

Numerous studies have shown negative effects of long-term khat use on testicular structure and function in humans and experimental animals. Damage to testicular structure is restricted to spermatogenic cells [9;10] and Leydig cells [11] while functional impairment is primarily that of endocrine nature [12,13,14,15]). Previous studies showed that khat extract's cathinone depresses cell proliferation and inhibition of RNA, DNA and protein synthesis in rapidly proliferating cells leading to impaired spermatogenesis and steroidogenesis [16,17]. In the wake of these adverse effects, the existence of recovery of function and structure has not been established yet.

It is clearly known that regulation of hypothalamo-pituitary-gonadal axis is controlled by a feedback loop system operating from gonads to the pituitary [18]. In this regard, we hypothesized that long-term khat use impairs gonadotrophic cell function that controls spermatogenesis. To test these hypotheses, the present study was conducted to further characterize endocrinological input on testicular structure and function associated with khat use withdrawal following sub-chronic exposure to provide information on the physiological link between long-term drug use and subsequent withdrawal and functional system regeneration, which forms the basis of our aim of study.

2. MATERIALS AND METHODS

2.1 Experimental Animals and Housing

Twenty-eight male sexually mature New Zealand white, albino, rabbits aged between 9 and 12 months and weighing about 1.5 to 2.5 kg were obtained from a breeding facility in the School of Biological Sciences, University of Nairobi, Nairobi. Animals were kept in the animal house situated at the same school where they were caged singly (16 inches x 16 inches x 13.3 inches) and beddings changed every three days of the week. The housing conditions entailed 10 hr light: 14 hr dark cycle and an average room temperature of 23°C with humidity of approximately 60%. All the experimental procedures were conducted during the light cycle. Food (commercial standard rabbit pellets,

Unga feeds, Nairobi) and fresh tap water were provided *ad libitum*. Carrots and green vegetables were also fed daily alongside as supplement diet.

2.2 Preparation of Crude Khat Extract

Fresh khat leaves were obtained from Nyambene Hills of Meru County, Kenya and wrapped in fresh banana leaves with the sole aim of preserving moisture and prevention of biodegradation of active biomolecules. Three doses (1 g/kg, 10 g/kg and 20 g/kg body weight) of khat extract were prepared from the fresh leaves blended in normal saline according to procedure earlier described [9]. Briefly, for a dose of 1 g/kg body weight, 10 g of fresh khat leaves were blended in 10 ml normal saline to yield a concentration of 1 g/ml. For a rabbit weighing 2 kg, 2 g of khat extract was used. Thus 2 ml of the stock solution containing 1 g/kg was mixed with 8 ml of normal saline to make a total volume of 10 ml. To prepare a dose of 10 g/kg body weight 100 g of fresh khat leaves were blended in 10 ml normal saline to yield a concentration of 10 g/ml. For a rabbit weighing 2 kg, 20 g of khat extract was used. Thus 5 ml of the stock solution containing 10 g/ml was mixed with 5 ml of normal saline to make a final volume of 10 ml. For 20 g/kg, 120 g of fresh khat leaves were blended in 6 ml normal saline to give a concentration of 20 g/ml.

2.3 Experimental Design and Khat Extract Administration

Animals were randomly assigned to 7 groups of 4 animals each with similar weight and age group I serving as control while group II, III and IV as test animals during treatment phase while group V, VI and VII serving as khat-treated followed by post-withdrawal observation. The sample size was determined according to the method earlier described [19] on animal studies using resource equation approach. The animals were habituated to handling and insertion of intra-gastric tube for about 20 min daily for 2 weeks while in restraint boxes (380 mm x 175 mm x 230 mm and neck slot of 220 mm) before commencement of experiments. Control animals were each administered 10 ml normal saline via oral gavage three times a week for 12 weeks while test animals in respective groups were administered 1 g/kg, 10 g/kg and 20 g/kg body weight of khat extract in 10 ml volume via oral gavage following the same regimen. The doses were chosen based on previous studies in rabbits [9], which indicated optimum effect of khat extracts on reproductive parameters to be within this dose range.

2.4 Blood and Tissue Sampling and Processing

At the end of treatment period, blood samples (1.5 ml) from each animal of each group was collected in heparinized LP_3 via marginal ear vein using needle (22G) and syringe for hormonal assays and consequently animals euthanized with Sagatal sodium (Phenobarbital sodium, Rhone Merieux Ltd, Lyon, France) at 26.4 mg/kg body weight *i.m* for testicular and epididymal tissue harvesting for histology. Group V, VI and VII animals were also treated with 1 g/kg, 10 g/kg and 20 g/kg body weight of khat extract in 10 ml volume via oral gavage and allowed 4 weeks post-withdrawal from sub-chronic khat extract exposure for investigation of recovery effects. Blood samples were collected in heparinized LP_3 vials, centrifuged at 1500 x g for 5 min and plasma stored at -20°C for hormonal analysis.

2.4.1 Hormonal analysis

Hormonal assays for plasma Follicle Stimulating Hormone and testosterone were done by use of enzyme immunoassay technique using the kits from Nova Tec Immun diagnostica GMBH, Germany. The technique uses the principle of competition of hormone in sample with enzyme conjugated hormone for limited binding sites on the specific antibody. Samples were first validated for use in rabbits before assays were done. In all hormone assay procedures, assays were done in triplicate. The optical density of specimen was measured using Huma Reader HS (Gessells chaft für Biochem und Diagnostica, mbH, Germany). The enzyme immunoassay for FSH and testosterone followed the assay procedures stipulated in the protocols supplied with the kits.

2.4.2 Tissue processing for histology

The histological tissue sampling and processing for examination was done as described earlier [20]. Briefly, euthanized animals were perfused intra-cardially with phosphate buffered saline followed by fixation using 10% (v/v) formaldehyde (pH 7.4). Testicular and epididymal tissues were carefully harvested, cut into sufficiently small sizes (1 mm³) to permit proper

fixation and processing and then immersed into the same fixative for 2 weeks to achieve maximum fixation. Thereafter, the tissues were dehydrated through ascending grades of ethanol (70%, 80%, 95% and twice in absolute ethanol), cleared using xylene and finally embedded in molten paraffin wax (56°C). Semi-thin sections (1 μm thick) were then obtained from tissue blocks using a rotary microtome (Leitz Wetzlar Company, Wetzlar, Germany), mounted on moistened glass slides, fixed and stained with Eosin and Hematoxylin. The tissue blocks were then dried and viewed under light microscope (Leica DMR 500; Leica Camera AG, Solms, Germany) fitted with a digital camera and a computer where photomicrographs taken and analysed.

2.5 Statistical Analysis

Hormonal data are presented as mean ± SEM. Data for treatment and post-treatment phases were analysed separately. For each hormonal measure, a 7 group (control, 1 g/kg, 10 g/kg, 20 g/kg body weight, 1 g/kg body weight + withdrawal period, 10 g/kg body weight + withdrawal period, 20 g/kg body weight + withdrawal period) x experimental period (12 weeks for treatment phase and 4 weeks for post-withdrawal phase) one-way ANOVA was conducted, using group as a between-subject factor and week as a within-subject factor. Multiple comparisons for mean differences among groups during treatment and post-withdrawal period was done using Tukey's post hoc test. Differences were considered significant at $P \leq 0.05$.

3. RESULTS

3.1 Effects of Khat Extract on Testicular and Epididymal Histology during Sub-chronic Exposure

3.1.1 Effects on testis

Results showed that medium (10 g/kg body weight) (Fig. 1C) and high dose (20 g/kg body weight) (Fig. 1D) of khat extract at sub-chronic exposure led to vacuolation in spermatogonia, spermatocytes and spermatids with accompanying pyknosis of nuclei indicative of cellular degeneration. The remarkable sloughing off of nuclei and germ cell vacuolation showed aberrations leading to impairment of spermatogenesis. However, animals at low dose (1 g/kg body weight) of khat extract showed no

appreciable observable lesions (Fig. 1B) on testicular histology when compared to control group (Fig. 1A). Sertoli cells appeared lying intimately on the basement membrane and projecting through the entire diameter of seminiferous tubular epithelium. At all doses of khat extracts, Leydig cells displayed structural characteristics similar to those observed in controls indicating a possible lack of histological alterations.

3.1.2 Effects on epididymis

At high dose of khat extract, there were no apparent structural alterations in corpus (Fig. 2A), cauda (Fig. 2B) and caput epididymis (Fig. 2C) when compared to controls (Fig. 2D). For instance, in the caput epididymis, the cytoplasm of principal cells contained prominent Golgi vesicles, numerous secretory granules, multi-vesicular bodies and apical stereocilia as those observed in control group. The lumen of epididymis contained spermatozoa indicative of normal function of storage and maturation processes. The arrangement of epididymal epithelia showed uninterrupted relationship with the basement membrane and the pseudostratified nature was intact for treatment groups compared to the control.

3.2 Effect of Khat Extract on Plasma FSH and Testosterone during Treatment Period

Plasma FSH levels were investigated to measure, in part, Sertoli cell activity since these are testicular cells that express the FSH receptors. The present study did not, however, consider receptor binding assays for FSH, which could have provided more insights into integrity of Sertoli cell function. Results showed that khat extract at medium and high doses suppressed plasma FSH with significant ($P<0.05$) decrease at high (1.72 ± 0.19) and medium (2.5 ± 0.05) doses compared to low dose group (3.4 ± 0.70) and control (6.61 ± 0.19) (Fig. 3). A similar pattern of effect was observed with measurements on plasma testosterone where high khat dose had a significant ($P<0.05$) suppressive effect (Fig. 4). The mean plasma testosterone levels recorded were 1.75 ± 0.55 for high dose, 1.96 ± 0.92 for medium dose and 3.02 ± 0.88 for low dose compared to 4.65 ± 0.4 for control group. The order of khat extract suppressive doses effect was high>medium>low>control on FSH and Testosterone.
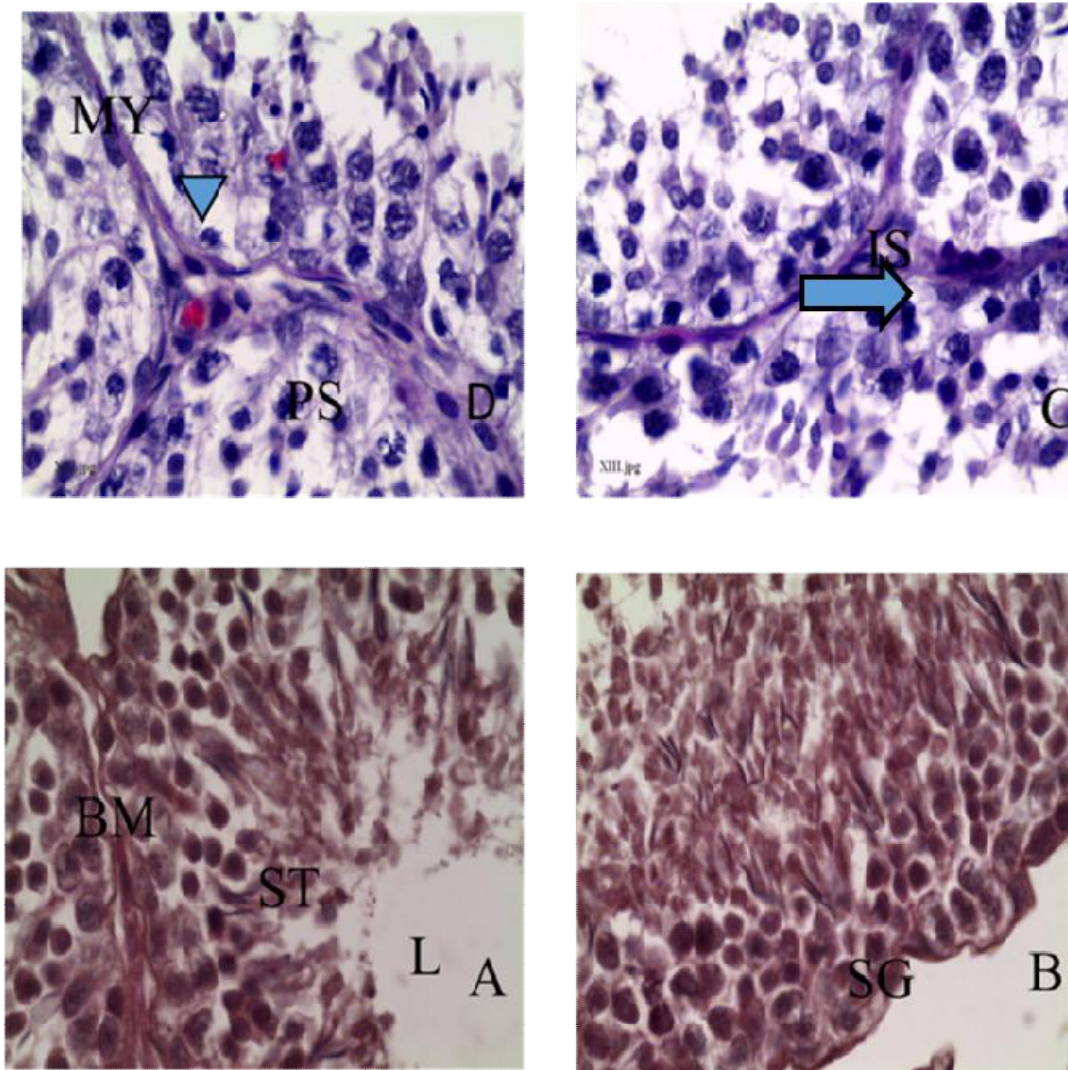
Fig. 1. Histology of the testicular tissues of saline-treated controls (A), low (B), medium (C) and high dose (D) of khat extract groups. Note the intact seminiferous epithelium at different stages of development with spermatogonia lying intimately on the basement membrane (BM) in control and at low dose group. Sub–chronic exposure of khat at medium and high dose showed pyknosis of nuclei in spermatocytes (arrow) with accompanying vacuolation and nuclei marginalization in spermatogonia (arrowhead). (Haematoxylin and eosin staining x400)

3.3 Testicular Histology at End of One-Month Post-withdrawal Period Following Sub-chronic Khat Extract Exposure

The results showed regeneration of seminiferous epithelium in damaged testicular tissues of animals that had been exposed to medium (Fig. 5C) and high (Fig. 5D) khat dose. The morphological picture was similar to those of animals that had been exposed to low dose (Fig. 5B) and controls (Fig. 5A) during treatment phase. The seminiferous tubules of testis showed various forms of spermatogenic cells at different stages of development lying on intact basement membrane alongside Sertoli cells with cytoplasmic processes projecting into the lumen, which appeared filled with spermatozoa. Evidence of seminiferous regeneration was supported by comparison of histological picture in control animals that did not receive any khat treatment but were taken through to the post-withdrawal period.
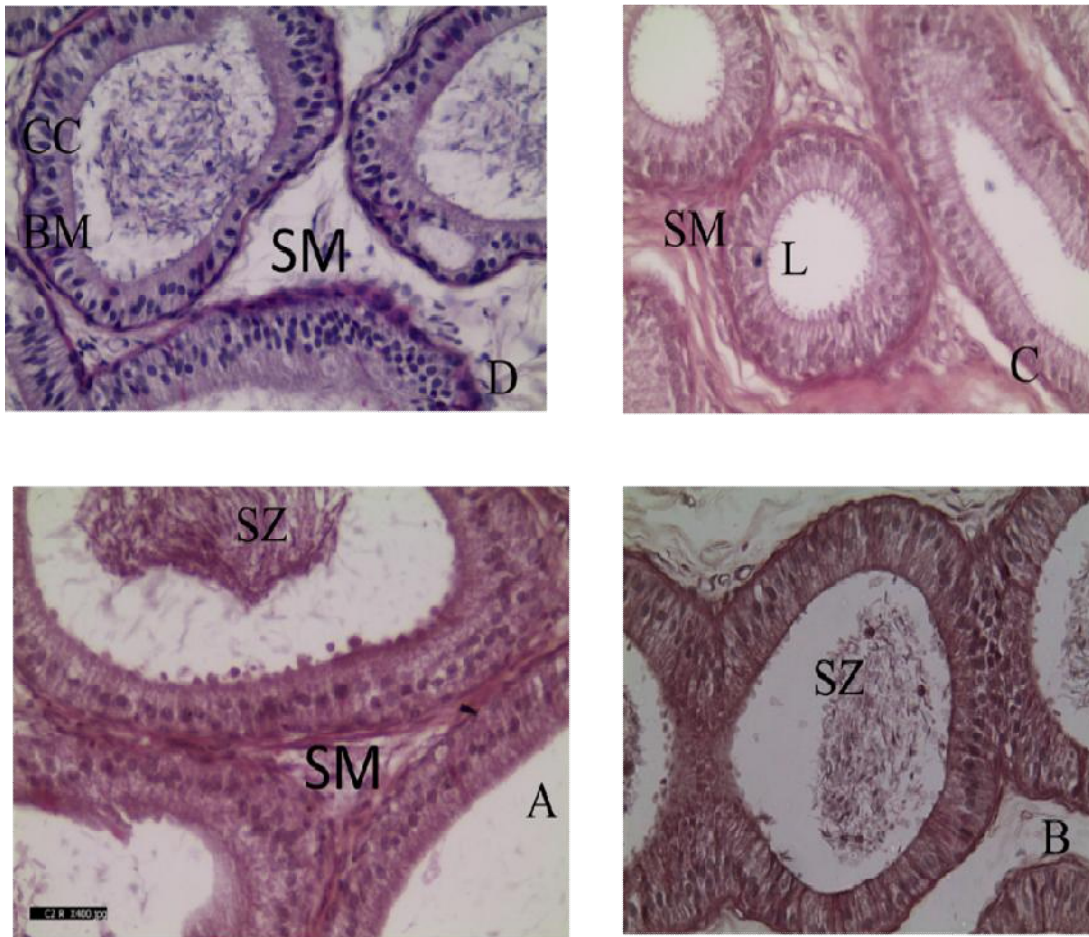
Fig. 2. Epididymides of rabbits exposed to high dose of khat extract for 12 weeks. There were no observable histological alterations on epididymides khat- treated animals. A) Corpus epididymis generally showing normal morphology with secretory granules, short apical villi and Golgi vesicles. B) Cauda epididymal epithelium showing epithelia resting on an intact basement membrane (BM) with short apical villi and lumen with spermatozoa (SZ). C) Caput epididymal epithelium of rabbit at high dose of khat extract for 12 weeks. D) Caput epididymal epithelium of control showing tall principal cells (CC) resting on basement membrane with stereocilia projecting into the lumen (L) and generally appear pseudostratified, characteristic of normal caput epididymis. The basement membrane of epididymides is covered by smooth muscles (SM). Haematoxylin and Eosin Stain x 400 magnification

3.4 Plasma FSH and Testosterone at the End of One- month Post-withdrawal Period from Sub Chronic Khat Extract Exposure

During withdrawal period, plasma levels of FSH that had been reverted to almost normal in rabbits exposed to low dose of khat was higher (5.8 ± 0.11) than those at medium (4.3 ± 0.07) and high dose (4±0.04) compared to controls (6.61 ± 0.03) (Fig. 6). A similar scenario was observed with results on plasma testosterone production in animals that had been subjected to sub-chronic exposure to khat treatment followed by one- month withdrawal period. The increment in testosterone levels post-withdrawal was significant ($P<0.05$) in animals that had been subjected to low dose compared to medium and high dose treatment groups (Fig. 7). The mean plasma testosterone during post-withdrawal period in animals that had been treated with low dose (4.1±0.88) was slightly higher than those at medium (3.42 ± 0.7) and high dose (2.25 ± 0.5) compared to the control (4.65 ± 0.44).
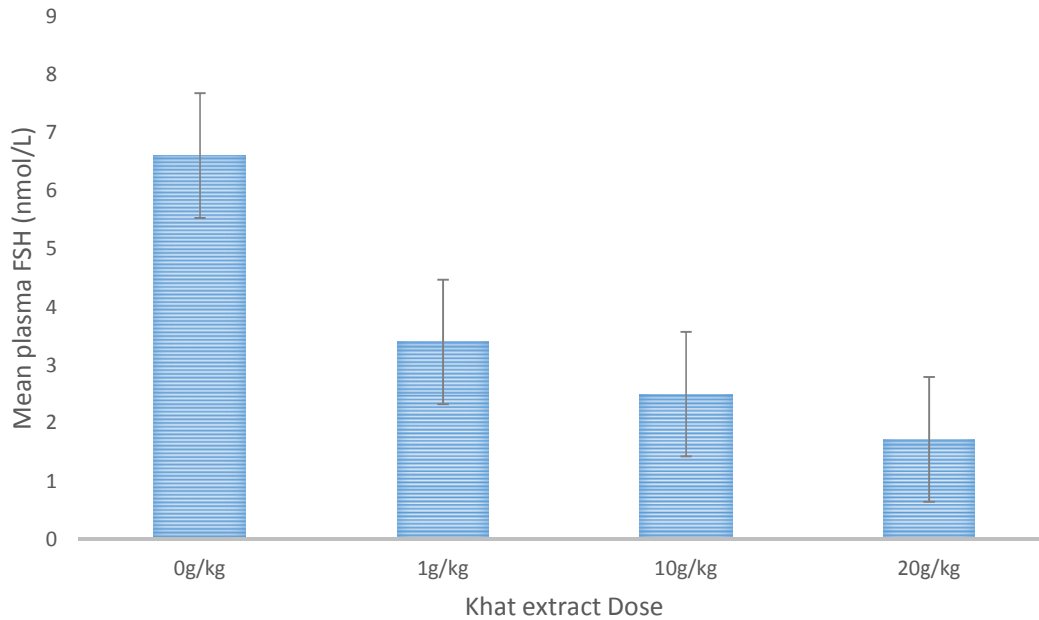
Fig. 3. Mean plasma levels of FSH in saline treated control and khat treated rabbits as a function of dose. There was a significant suppression (*P*<0.0) of plasma FSH levels at medium and high dose of khat extract compared to control group; n = 28
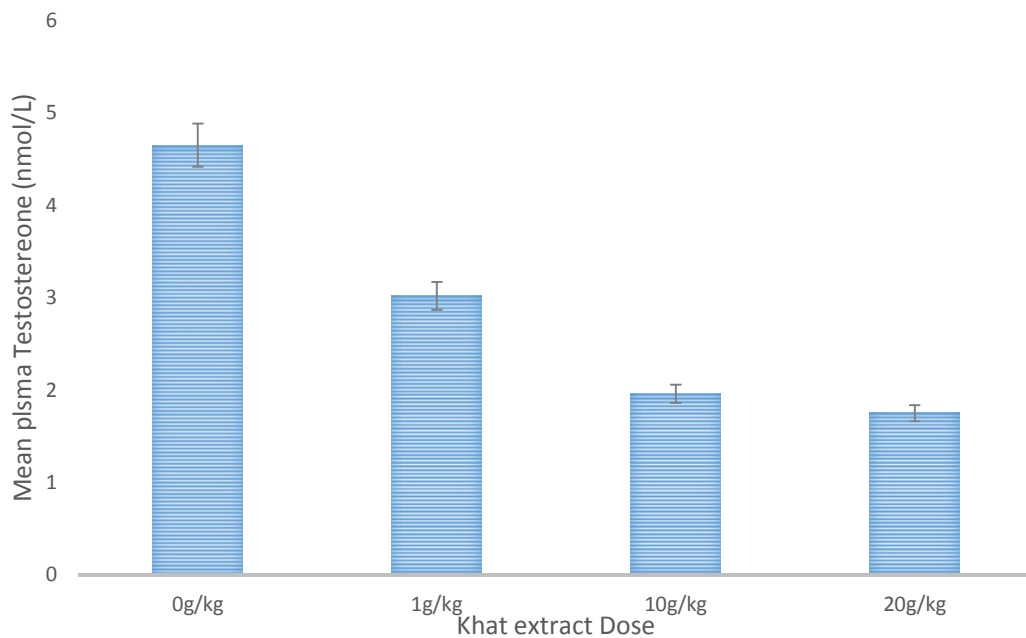

Fig. 4. Figure showing pattern of mean plasma testosterone levels following sub-chronic khat exposure to rabbits at different dose levels. There was a significant decrease (*P*<0.05) in plasma testosterone in animals at medium and high dose of khat compared to the control; n = 28
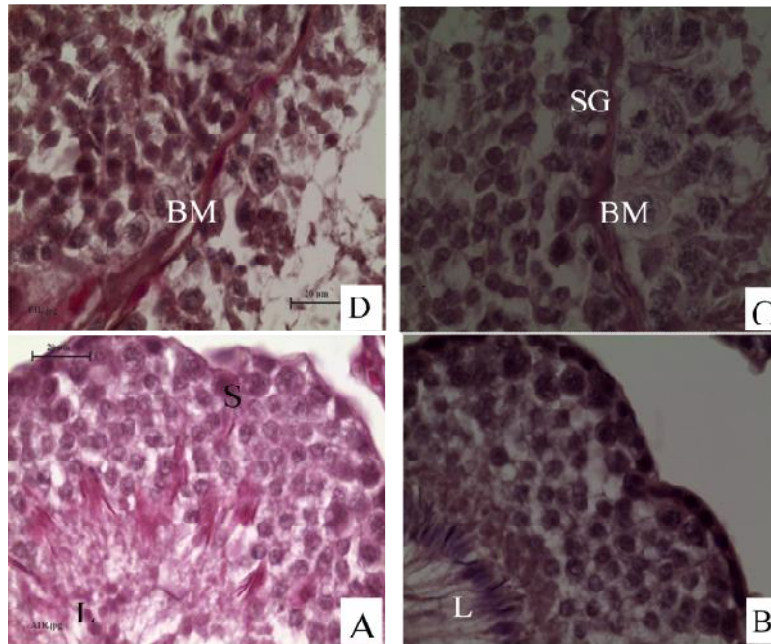
Fig. 5. Histology of rabbit testis at the end of one- month post-withdrawal showing control (A), animals that had been exposed to low dose (B), Medium (C) and high dose (D) of khat extract. Note the normal architecture of seminiferous epithelium at different stages of development, with Sertoli cells (S) and spermatogonia (SG) lying on intact basement membrane (BM) in all groups of animals and spermatozoa in the lumen (L) in some cases. (Haematoxylin and Eosin Stain x 400)
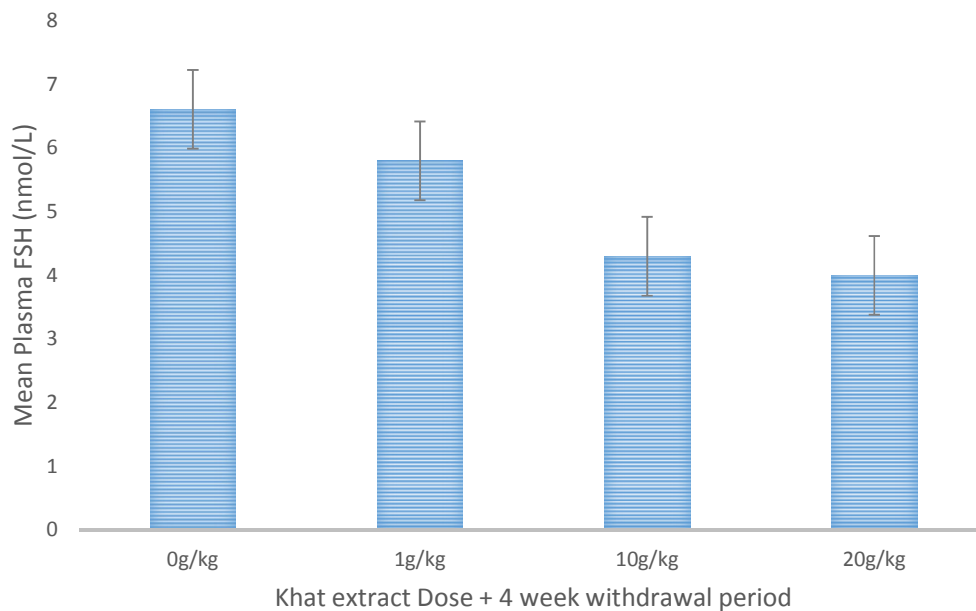

Fig. 6. Plasma FSH during withdrawal phase from sub-chronic exposure of khat extract to rabbits. Note that there was no significant different in plasma levels of testosterone among treatment groups that was observed during exposure phase. High and medium dose groups showed recovery of hormonal secretion post-withdrawal from khat extract exposure; n=8
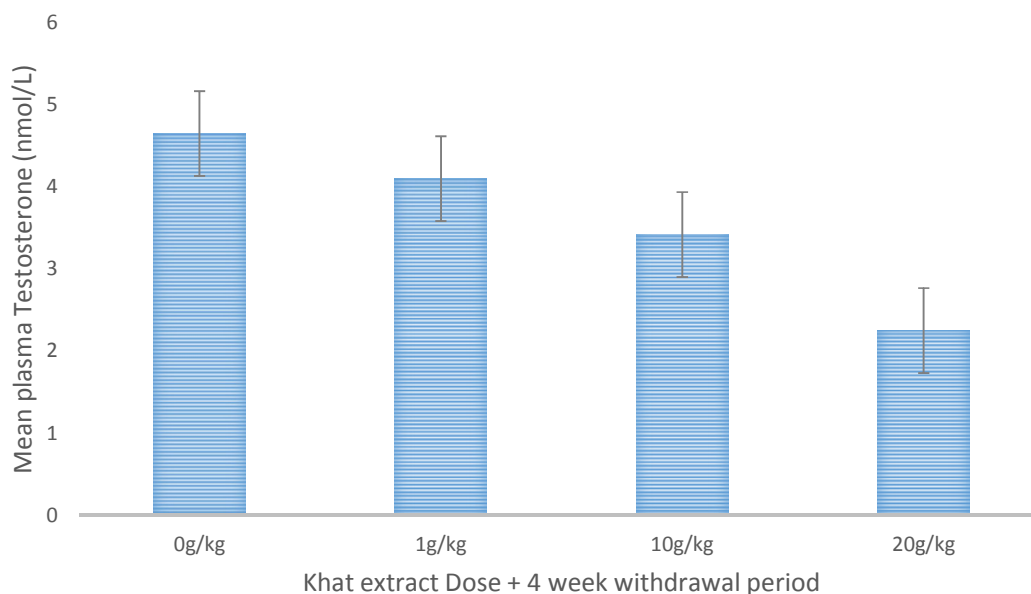
Fig. 7. Figure showing pattern of plasma testosterone levels following a 4-week withdrawal from sub-chronic khat extract treatment. There was observable recovery of testosterone production at all khat doses when compared to the control. The significant increase ($P<0.05$) in plasma testosterone was recorded for medium and high dose groups; n =16

4. DISCUSSION AND CONCLUSION

The major aim of this study was to ascertain the possible recovery of testis during post-withdrawal period from testicular function damage owing to long-term exposure to khat extract. The study considered effects of khat use on reproductive parameters of plasma FSH and testosterone as well as histological evaluation of testis and epididymis during sub-chronic khat extract exposure and followed the investigation on same parameters post- withdrawal to assess for potential regeneration of structure and function. Our results demonstrate that spermatogenesis and steroidogenesis as assessed by testicular and epididymal histology alongside plasma FSH and testosterone may be re-initiated and maintained to normal levels following withdrawal from long-term khat use. Majority of the reports published to date on khat use and misuse have focussed entirely or almost entirely on effects during heavy and long-term use and accompanying withdrawal syndrome. Most of these studies have reported central nervous system effects including euphoria, excitation, anorexia, increased respiration, hyperthermia, logorrhoea, analgesia and increased sensory stimulation [21], reproductive function impairment in rats [14], humans [10] and rabbits [9] and accompanying withdrawal symptoms of lethargy, mild depression, slight trembling and recurrent nightmares [22].

The results of the present study showed regeneration of spermatogenic cells specifically spermatogonia, spermatocytes and spermatids at end of 1- month withdrawal period following cytotoxic damage from sub-chronic exposure. The mechanism of action of subsequent regeneration of spermatogenic cells post-withdrawal of khat following destruction during sub-chronic exposure is not clear. This finding of the present study is significant since it is the first of its kind to shed light into the potential recovery from hazardous effects owing to khat use and misuse. Spermatogenic cell regeneration was reported in earlier studies [23], which showed that colchicine impairs spermatogenesis through disruption of microtubules thus interfering with mitosis and meiosis. Although study focus was different from that of the present study, studies in mouse showed that disruption of microtubules using vinblastine and vincristine causes degeneration in differentiating spermatogonia but not stem cell spermatogonia [24,25]. In rodent studies, spermatogeneic stem cell has been identified as an isolated, undifferentiated, type A spermatogonium [26]. These cells play role both

for steady state spermatogenesis and for regeneration following cytotoxic [27]. In our study, we were not able to identify stem cell spermatogonia in histological sections hence we can only speculate that long-term khat use probably impaired stem cell spermatogonia differentiation into proliferating spermatogonia. It would appear that long-term khat use is detrimental at the level of Leydig cell and seminiferous tubule. Although the seminiferous epithelial cycle was not keenly considered in this study, it appears khat preferentially affected germ cell spermatogonia and subsequent daughter cells. This is evident by vacuolation in spermatogonia and spermatocytes in animals at high dose of khat extract. Meistrich [25] reported that differentiating spermatogonia have a high labelling index and proliferation following cytotoxic treatment and that acute effect of drugs target most sensitive germ differentiating spermatogonia. In a related study in mice, 4'-(9-acrinylamino) methanesulfon-o-anisidide was shown to preferentially kill differentiating spermatogonia with no apparent effect on stem cell spermatogonia [28]. The reversal effects at end of post-withdrawal from long-term khat use point towards stem cell spermatogonia regeneration that gave rise to more germ cell spermatogonia hence recovery of spermatogenesis. The mechanism to this recovery is not very clearly understood. However, a related earlier study in human subjects showed that cisplatin and concomitant treatment with testosterone caused recovery from germ cell loss [29]. Although the present study did not consider investigation into the involvement of stem cell factor expression, it is possible that lack of stem cell spermatogonia damage may have involved a changing pattern of stem cell factor expression following long-term khat use. Studies by Udagawa et al. [30] on rats using leuprorelin acetate showed that ratio of expression of membrane-bound stem cell factor to soluble stem cell factor was increased. We could not convincingly argue our findings towards this direction and therefore offers a knowledge gap for further investigations.

In the present study, during sub-chronic khat exposure to rabbits, there was no apparent histological changes on Leydig cells. However, the same animals showed dose-dependent decrease in plasma testosterone. We could not report cytological details since the study did not consider transmission electron microscopic evaluation yet hormonal data from same study subjects indicated a significant effect on testosterone levels for high dose of khat extract at sub-chronic exposure, and this possibly contributed to the limitations of the study. Quite recently, it was reported that long-term exposure to khat interferes with steroidogenesis by impairing structural integrity of mitochondria, lipid droplets and smooth endoplasmic reticulum in Leydig cells [11]. The results at end of post-withdrawal period showed recovery of testosterone production to pre-treatment values in treatment groups when compared to the control group. Since gonadal steroidogenesis is restricted to involvement of smooth endoplasmic reticulum [31] and lipid droplets [32], recovery of function post-withdrawal possibly involved regeneration of these structures. Nonetheless, it may be possible that effect of khat on testicular function is not restricted to Leydig cells and spermatogenic cells but also indirectly through Sertoli cells. Sub-chronic exposure to rabbits in the present study showed no apparent histological changes on the structural integrity of Sertoli cells when compared to control animals. When taken through the withdrawal period, the same histological picture was observed for animals across all groups. Sertoli cells have been shown to provide critical support required for germ cell differentiation [33]. Interestingly, hormonal results in the same set of animals showed a dose-dependent suppression of plasma FSH during sub-chronic khat extract exposure. This result compromised the theoretical foundation of the hypothesis that suppression of gonadal function through negative feedback mechanism increase rate of FSH synthesis by pituitary gland gonadotrophs. This is particularly so if the Sertoli cells are unaffected, like is the case in the present study. The FSH results also contradict findings in velvet monkeys treated with cathinone at long-term regimen and which reported upregulation of labelling index of gonadotropic cells [12]. The study attributed the increase of gonadotropic activity to changes in intracellular signalling and specifically cyclic AMP, cyclic GMP, arachidonic acid and changes in lipoxygenase pathway as earlier reported [34]. The contradiction in findings is not very clear although all point towards a link between FSH signalling and testicular function.

The involvement of FSH in male reproduction is not clearly explained and this has led into controversial reports. On the one hand, it has been shown that FSH plays a significant role in reproduction [35] through interaction with FSH receptor [36]. In male animals, FSH receptor has been expressed in Sertoli cells [37] and is

believed to be essential for initiation and maintenance of spermatogenesis [38]. On the other hand, it is reported that response of FSH to Sertoli cell spermatogenesis is more pronounced in young animals [39] and less so as the animal matures. This mechanism was attributed to an increase in the phosphodiesterase activity in cells and subsequent accumulation of cAMP that led to compromised protein synthesis [40]. The latter observation was supported by Dierich et al. [41] who reported that although FSH signalling is not essential for initiation of spermatogenesis, it appears essential for adequate viability and motility of sperm. Gonadotropin releasing hormone-deficient mutant mice treated with testosterone implants had normal spermatogenesis but decreased testis size and germ cell numbers [42] and, with FSH treatment resulted in normal spermatogenesis with quantitatively normal size [43]. In the present study, long-term khat exposure possibly selectively interfered with gonadotrope function owing to the sexually mature rabbits used for the study. This, however, remains speculative since studies did not compare effect of age on gonadotropic cell response to long-term khat extract exposure. Further, the results of post-withdrawal period showed increase in plasma FSH to pre-treatment values when compared to control. This reversal effect is a pointer that long-term effect of khat extract probably involved FSH receptor on Sertoli cells, which led to impairment of function and following withdrawal there was accompanying restoration of function of these structures. This was evident by increase in plasma testosterone in same animals. Sertoli cells express the androgen binding protein and localizes the androgens close to dividing germ cells hence successful spermatogenesis [44].

The spermatogenic recovery after damage following long-term khat use in the present study is gaining increasing support from previous studies [45,46]. Previous studies have also demonstrated that proliferation of germ cells is normally excessive and a tightly regulated system of physiological apoptosis, which optimizes the output of germ cells to a level sustainable by seminiferous epithelium. In this regard, brief exposure to testicular toxicant or less severe, the seminiferous epithelium can often recover and rapidly re-establish normal spermatogenesis [47]. The results of the present study certainly favour this proposition and probably categorize what was regarded as sub-chronic exposure of khat extract to animals to have been less severe to cause irreversible

effects on testicular structure and function. The caput, corpus and cauda epididymis showed no apparent structural alterations during sub-chronic khat extract exposure and so studies were not followed at post-withdrawal period.

The study had its own limitations, which included the need for receptor binding assays and resources to perform ultrastructural studies of testicular cells. Transmission electron microscopic examination could have offered information on sub-cellular organelle involvement in drug-function interaction. Follicular stimulating hormone receptor expression on Sertoli cells could have offered insights into the extent of khat effects on spermatogenic function of Sertoli cells. Results have also pointed to the possible role of stem cell spermatogonia in restoration of spermatogenesis. Identification of stem cell spermatogonia and evaluation of stem cell expression factor could have given more insights into the mechanism of spermatogenic recovery observed. Together, these limitations form a concrete knowledge gap for further research especially on the mechanistic link between cytotoxic germ cell effects of long-term khat exposure germ cell recovery post-withdrawal from long-term khat use. However, overall, the findings of the present study have demonstrated, for the first time, the evidence of testicular function recovery following damage from long-term khat exposure. This finding is significant since it provides insights to public health practitioners and policy makers on post-withdrawal effects following long-term khat use.

HIGHLIGHTS

- Chronic and heavy khat use causes cellular degeneration in different tissues and organs of the body
- Rabbits offer excellent animal model for studies on reproductive function in addictive cases and hormonal measurements
- Germ cell degeneration following long-term exposure to noxious cytotoxic agents undergoes regenerative cycle following a window of withdrawal from use.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not

intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ETHICAL APPROVAL

ACKNOWLEDGEMENTS

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Orth J, Christensen AK. Autoradiographic localization of specifically bound ^{125}I-labeled follicle-stimulating hormone on spermatogonia of rat testis. Endocrinology. 1978;103:1944–1951.
2. Means AR, Fakundig JL, Huckins C, Tindall DJ, Vitale R. FSH, the Sertoli cell and spermatogenesis. Recent Progress in Hormone Research. 1976;32:477–525.
3. Dorrington JH, Armstrong DT. FSH stimulates estradiol-17β synthesis in cultured Sertoli cells. Proceedings in National Academy of Sciences, NY, USA. 1975;72:2677–2681.
4. Bremner WJ, Matsumoto AM, Sussman AM, Paulsen CA. Follicle-stimulating hormone and human spermatogenesis. Journal of Clinical Investigation. 1981;68: 1044–1052.
5. Hall PF. Testicular steroidogenesis organization and regulation. In: Knobil E, Neill JD (Eds). The physiology of reproduction, second edition. Ravons Press, New York. 1994;1335–1362.
6. Akingbemi BT, Ge RS, Klinefelter GR, Gunsalus GL, Hardy MP. A metabolite of methoxychlor, 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane, reduces testosterone biosynthesis in rat leydig cells through suppression of steady-state messenger ribonucleic acid levels of the cholesterol side-chain cleavage enzyme. Biology of Reproduction. 2000;62:571–578.
7. Marques-Pinto A, Carvalho D. Human infertility: Are endocrine disruptors to blame? Endocrine Connections. 2013;2: 15–R29.
   DOI: 10.1530/EC-13-0036.
8. Cox G, Rampes H. Adverse effects of khat: A review. Advances in Psychiatry Treatment. 2003;9:456–463.
9. Nyongesa AW, Patel N, Wango EO, Onyango DW. High khat dose and long-term exposure impairs spermatogenesis: experimental study using rabbit model. Journal of Morphological Sciences. 2017; 34:156–167.
10. El-Shoura SM, Abdel Aziz M, Elmalik EM. Deleterious effects of khat addiction on semen parameters and sperm ultrastructure. Human Reproduction. 1995; 9:2295-2300.
11. Nyongesa AW, Oduma JA, Okelo DO, Chirwa S. Khat (*Catha edulis*) addiction, effects on general body health and interventional remedial measures. In: Addiction and Recovery E-books. 2019; 1(4):1–18.
12. Nyongesa AW, Oduma JA, al'Absi M, Chirwa S. Immunohistochemical localization of anterior pituitary cell types of vervet monkeys (*Chlorocebus aethiops*) following sub-chronic cathinone exposure. Journal of Ethnopharmacology. 2015;174: 168–177.
13. Nyongesa AW, Onyango DW. Khat: A boon or bane to humanity. In: Awaad AS, Singh VK, Govil JN (Eds). Recent Progress in Medicinal Plants. Studium Press LLC, USA. 2010;28:188–209.
14. Mohammed A, Engidawork E. Reproductive parameters are differentially altered following sub-chronic administration of *Catha edulis* Forsk (khat) extract and cathinone in male rats. Journal

of Ethnopharmacology. 2011;134:977–983.

15. Abdul waheb M, Makonnen E, Debela A, Abeba D. Effect of *Catha edulis* Forsk (khat) extracts on male rat sexual behaviour. Journal of Ethnopharmacology. 2007;110:250–256.

16. Al-Meshal IA, Qureshi S, El-Feraly FS, Tariq M. The toxicity of *Catha edulis* (khat) in mice. Journal of Substance Abuse. 1991;3:107-115.

17. De Hondt HA, Fahmy AM, Abdelbaset SA. Chromosomal and biochemical studies on the effect of khat extract on laboratory rats. Environmental Mutagenesis. 1984;6:851-860.

18. Simoni M, Gromoll J, Nieschlag E. The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology and pathophysiology. Endocrinological Reviews. 1997;18:739–773.

19. Arifin WN, Zahiruddin WM. Sample size calculation in animal studies using resource equation approach. Malaysian Journal of Medical Sciences. 2017;24:101–105.

20. Ito S, Karnovsky MJ. Formaldehyde-glutaraldehyde fixatives containing trinitro compounds. Journal of Cell Biology. 1968; 39:168–169.

21. Nencini P, Ahmed AM. Khat consumption: A pharmacological review. Drug and Alcohol Dependence. 1989;23:19–29.

22. Kalix P. Khat: A plant with amphetamine effects. Journal of Substance Abuse and Treatment. 1988;5:163–169.

23. Russell LD, Malone JP, MacCurdy DS. Effect of the microtubule disrupting agents colchicine and vinblastine on the seminiferous tubule structure in the rat. Tissue and Cell. 1981;13:349.

24. Meistrich ML. Quantitative correlation between testicular stem cell survival, sperm production and fertility in the mouse after treatment with different cytotoxic agents. Journal of Andrology. 1982;3:58.

25. Meistrich ML. Relationship between spermatogonial stem cell survival and testis function after cytotoxic therapy. British Journal of Cancer. 1986;53(7):89–101.

26. Huckins C, Oakberg EF. Morphological and quantitative analysis of spermatogonia in mouse testes using whole mounted seminiferous tubules. II. The irradiated testes. Anatomical Record. 1978;192:529 – 542.

27. Clermont Y, Hermo L. Spermatogonial stem cells in the albino rat. American Journal of Anatomy. 1975;142:159-175.

28. Da Cunha MF, Meistrich ML, Finch-Neimeyer MV. Effects of AMSA, an antineoplastic agent, on spermatogenesis in the mouse. Journal of Andrology. 1985; 6:225-229.

29. Vawda AI. Effect of testosterone on cisplatin-induced testicular damage. Archives of Andrology. 1994;32:53–57.

30. Udagawa K, Ogawa T, Watanabe T, Yumura Y, Takeda M, Hosaka M. GnRH Analog, leuprorelin acetate promotes regeneration of rat spermatogenesis after severe chemical damage. International Journal of Urology. 2001;8:615–622.

31. Christensen AK. Leydig cells. In: Greep RO, Astwood EB. (Eds.). Handbook of Physiology. American Physiological Society: Washington DC. 1975;5(7):57.

32. Moses HL, Davis WW, Rosenthal AS, Garren LD. Adrenal cholesterol: Localization by electron microscope audioradiography. Science. 1969;163: 1203-1205.

33. Parvinen M. Regulation of the seminiferous epithelium. Endocrinological Reviews. 1982;3:404–417.

34. Dan-Cohen H, Sofer Y, Schwartzman ML, Nataraja RD, Nadler JL, Naor Z. Gonadotropin releasing hormone activates the lipoxygenase pathway in cultured pituitary cells: role in gonadotropin secretion and evidence for a novel autocrine/paracrine loop. Biochemistry. 1992;31:5442–5448.

35. Ulloa-Aguirre A, Midgley AR, Beitins IZ, Padmanabhan V. Follicle-stimulating hormone: characterization and physiological relevance. Endocrinological Reviews. 1995;16:765–787.

36. Sprengel R, Braun T, Nikolics K, Segaloff D, Seeburg PH. The testicular receptor for follicle-stimulating hormone: Structure and functional expression of cloned cDNA. Molecular Endocrinology. 1990;4: 525–530.

37. Rannikki AS, Zhang FP, Huhtaniemi I. Ontogeny of follicle-stimulating hormone receptor gene expression in the rat testis and ovary. Molecular and Cellular Endocrinology. 1995;107:199–208.

38. Hammouda ES. Effect of khat extract, D-cycloserine and thiadomide on RNA of the neurons of 7 and 19 days of chick embryo. Bulletin of Sciences. 1971;64.

39. Fritz KB, Rommerts FG, Louis BG, Dorrington JH. Regulation by FSH and dibutynyl cyclic AMP of the formation of androgen-binding protein in sertoli cell-enriched cultures. Journal of Reproduction and Fertility. 1976;46:17–24.

40. Griswold MD. Action of FSH on mammalian sertoli cells. In: Griswold MD, Russell LD, (Eds). The sertoli cell. Clearwater FL: Cache River Press. 1993; 493–508.

41. Dierich A, Sairam RA, Monaco L, Fimia GM, Gansmuller A, Lemmeur M, Sassone-Corsi P. Impairing follicle stimulating hormone (FSH) signaling *In vivo*: Targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proceedings of the National Academy of Sciences, NY, USA. 1998;95: 13612–13617.

42. Singh J, O'Neill C, Handelsman DY. Induction of spermatogenesis by androgens in gonadotropin-deficient (hpg) mice. Endocrinology. 1995;136:5311–5321.

43. Singh J, Handelsman DJ. Neonatal administration of FSH increases sertoli cell numbers and spermatogenesis in gonadotropin-deficient (hpg) mice. Journal of Endocrinology. 1996;151:37–48.

44. Hiller-Sturmhöfel S, Bartke A. The endocrine system: An overview. Alcohol Health and Research World. 1998;22:153–164.

45. Meistrich ML, Wilson G, Huhtaniemi I. Hormonal treatment after cytotoxic therapy stimulates recovery of spermatogenesis. Cancer Research. 1999;59:3557–3560.

46. Shuttlesworth GA, De Rooij DG, Huhtaniemi I, Reissmann T, Russell LD, Shefty G, Wilson G, Meistrich ML. Enhancement of A Spermatogonial proliferation and differentiation in irradiated rats by gonadotropin-releasing hormone antagonist administration. Endocrinology. 2000;141:37–49.

47. Boekelheide K. Mechanisms of toxic damage to spermatogenesis. Journal of National Cancer Institute Monographs. 2005;34:3–8.