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Reproductive parameters in a 90 day toxicity study of Smart Herbal Purifier®-a poly herbal supplement in male rats

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ABSTRACT

The study was to evaluate the testicular and epididymal effects of Smart Herbal Purifier® (SHP) - a poly herbal supplement with a composition of *Cassia alata* 30 %, *Morinda Lucida* 30 %, and *Nuclea Blend* 40 % , in a 90 day repeated dose toxicity test in male Sprague-Dawley(SD) rats. The study was with control,48,240 and 480 mg/kg SHP treated rats. Sperm motility, debris generation, abnormal sperm morphology, sperm viability, testosterone, Follicular stimulating Hormone (FSH), luteinizing Hormone (LH), Prolactin, and malondialdehyde (MDA) levels as a measure of oxidative stress in the testes were analyzed. Histology of the testes was also examined. There was no significant difference in both Testicular Sperm Number (TSN), Epididymal sperm Number (ESN), the abnormal sperm morphology of both the control and SHP treated groups. A significant increase was observed in the debris generated in the testes and epididymis when control was compared to SHP treated groups. There was also a significant decrease in viability and motility in the testes and epididymis when control was compared to SHP treated group. Testosterone, FSH, LH and Prolactin were not significantly different when control was compared to SHP treated group. MDA was significantly increased when control was compared to the treated groups. Histology of the testes of the treated group showed necrosis of the seminiferous tubules. SHP may have toxicological concerns in the male reproductive system.

INTRODUCTION

Herbal supplements in various dosage forms namely tablets, capsules, syrups, suspensions etc, are often sold alongside conventional over-the-counter medications in retail outlets in Nigeria. Vendors of these herbal supplements are heavily patronised and the quest for alternative medicine has led to the official registration of these products. Unlike what is obtainable elsewhere, there are claims that these are intended to treat, cure, mitigate, prevent many diseases, and as substitutes for conventional medications (Shahrokh *et al*., 2005). Numerous products are currently being promoted for enhancing erectile function and sexual performance in men and are marketed with the implied assumption that they are safe and natural. Notwithstanding, herbal supplement contamination in Nigeria has been documented (Obi *et al*., 2006). SHP capsule is a poly herbal encapsulated product, registered in Nigeria and sold without prescription. In the language of the manufacturers SHP capsule, is an 'immune booster, sperm booster and a general body tonic. Cases of reproductive failure after prolonged intake of herbal supplements have been anecdotally reported in Nigeria. An increasing number of cases remain undocumented due to poor record keeping in the developing world (Orisakwe *et al*., 1996). Preliminary result of an ongoing study in Nigeria showed that 83.97% of the patients with history of herbal intake had abnornal seminal fluid analysis while only 16.03% of subjects with no history of herbal intake had abnormal result (Enuh *et al*., 2012). Although there is a large database of literature regarding the herbal drugs and their biological activity, what is disappointedly lacking is a systematic evaluation of their efficacy, safety and quality (Buttar & Jones, 2003). Considering the complexity of herbal products in general and their inherent biological variation, it is now necessary to evaluate their safety and quality (Castrol *et al*., 2009). It is feared that the chronic use of SHP may be implicated in some undocumented cases of male reproductive failure. This study evaluates the testicular and epididymal actions of SHP in SD rats.

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MATERIALS AND METHODS

Collection of sample

SHP was purchased in June 2012 from the manufacturer's outlet in Port-Harcourt, Nigeria.

Experimental animals

SD rats weighing 140-180g were obtained from the Faculty of Veterinary Medicine, University of Nigeria Nsukka. Animals were housed singly under standard laboratory conditions (ambient room temperature, 12 hr high/dark cycle) and all had free access to standard rat chow (Top Feeds Premier Feeds Flour Mills Nig. Plc., Lagos State) and tap water.

All animal experiments were conducted in accordance with internationally accepted practice for laboratory animal and approved by the Animal Ethics Committee of our University.

Experimental design

Weight matched rats, were divided into four groups of 30 animals each. The animals were housed singly. The test groups 2, 3 and 4 received 48, 240 and 480 mg/kg of SHP incorporated into the feed while the control, group 1 did not receive the SHP. To ensure that the test groups ingested the exact dose of SHP, the test groups received the SHP mixed with 5g feed firstly, prior to ad libitum feeding for the day.

The animals were observed for signs of toxicity and mortality throughout the experimental 90 days and the 30 day recovery period. The feed and fluid intakes were taken daily, while body weight was taken weekly. At the end of 30, 60, 90 days, 5 rats from each group were sacrificed under ether anesthesia. In the 30-day post treatment experiment, SHP was withdrawn from the test groups 2-4 but had access to standard rat chow and water. At the end of the withdrawal study, rats were also sacrificed under ether anesthesia.

Organ harvest

The testis were harvested and freed from adherent fats and the wet weight taken.

Sperm harvest and analysis.

The sperm analysis (motility, count, debris and abnormal morphology) was carried out. Briefly, laparatomy was done to expose the reproductive tract. The caudal epididymis was carefully isolated and minced with scissors to release the sperm into phosphate–buffered saline media and used for spermatological studies.

Sperm count

Each chamber of the haemocytometer was loaded with 10 µl of the diluted sperm (1:20 dilution) and allowed to stand and or settled for 5 min. Counting was done under a light microscope at 400× magnification.

Abnormal sperm morphology

Sperm morphology was determined using the eosin and nigrosin stain. Ten microlitres of eosin and nigrosin was mixed with about 40 µl of sperm suspension. The sperm suspension was incubated at 40 ◦C for 5 min and re-suspended with a micro-pipette. About 200 sperm cells per rat were morphologically examined under microscope at 400X magnification. Morphological abnormalities were classified as headless sperm, banana head, bent neck and bent tail. Sperm motility was done by placing 10 µl of sperm suspension on slide for microscopic evaluation at a magnification of 400x. About 200 sperm cells were examined and classified as either motile or immotile. The assessment of the motile sperm was calculated as mean motile sperm number ×100/total number of sperm.

Testicular and epididymal sperm motility

The testis and epididymis were excised and put on a petri dish containing modified Tyrode's medium prepared according to Fraser (1984). The testis and cauda epididymis was disrupted through an incision with a scissors previously wrapped with paraffin , and was kept for 30 min at 35 °C to promote the release of sperm into the medium. Then, sperm were collected with a micropipette (aliquot 20µl) to a slide for motility analysis. Sperm motility was determined by counting all progressive sperm, the non-progressive and the immotile sperm in the same field. In each preparation at least 100 sperm was counted.

Estimation of testis malonaldehyde (MDA)

Measurement of the activity of serum MDA (malondialdehyde) levels were done according to standard procedures: MDA EC 202-974-4 (Gutteridge&Wilkins ,1982)

Hormone analysis

The analysis of the hormones LH, FSH, testosterone and prolactin was carried out using automated microplate analyser.

Histopathological examination

The testes were prepared and stained with hematoxylin and eosin (H&E) staining technique, for photo microscopic observation.

Statistical Analysis

The data are expressed as mean ± SEM. Data were analysed using One way analysis of variance (ANOVA) and Mann Whitney U tests using (Graph Pad Prism version 5). Significance was determined at $p \leq 0.05$.

RESULTS

Effect of SHP on the body weight,testes weight, feed and fluid intake, and feed efficiency

The effect of SHP on the body weight, feed and fluid intake, and feed efficiency is shown in Table 1. There were no significant changes observed in the body weights, as every treatment group and control gained weight progressively.

However, the percentage weight gain of the control at each timeline was higher than that of the treatment groups. Also

the feed efficiency of the control group was higher. Although there was no significant difference in the fluid consumption, the group 4 animals had the highest mean consumption of fluid ($p<0.05$) when compared to the control. The absolute and relative weight of the testes of treated rats were not significantly affected by the ingestion of the SHP.

Effect of SHP on the testicular and epididymal sperm count, motility, abnormal sperm morphology, debris and viability

The effect of SHP on the testicular and epididymal sperm count, motility, morphology, debris and viability is shown in Table 2. Administration of SHP did not significantly affect the testicular sperm count but significantly decreased the epididymal sperm count. Testicular sperm motility significantly decreased from 71.87±12.98% (control group) to 27.13±6.64% (480 mg/kg SHP) treated group. Similar significant reduction (70.67±8.33 in control untreated group to 22.0±4.73 in 480 mg/kg SHP) in motility was seen in the epididymal sperm. There seem to be a non-significant change in the sperm morphology following ingestion of SHP. There was significant percentage increase in both testicular (19.10±5.78 in control untreated group to 57.50±6.89 in 480 mg/kg SHP) and epididymal (14±0.00 in control untreated group to 50.0±1.53 in 480 mg/kg SHP) sperm debris. In the same vein the percentage sperm viability decreased significantly from 73.50±9.55 to 26.07±1.98 for the control untreated and 480 mg/kg SHP treated groups respectively for the testicular sperm. The same pattern of percentage increase in sperm debris was seen in the epididymal sperm after SHP administration, in a dose dependent manner.

Effect of SHP on the serum levels of testosterone, LH, FSH and prolactin

The mean serum levels of testosterone, LH, FSH and prolactin are shown in Table 2. Ingestion of SHP significantly ($p<0.05$) increased FSH from 0.82±0.45 to 1.24±0.93 (Iμ/L) in the control untreated and the 240 mg/kg SHP treated groups respectively.

However the FSH level decreased to 0.68±0.41 (Iμ/L) at 480 mg/kg of SHP. The LH was significantly increased by 48 mg/kg SHP from 0.53±0.07 in the control untreated group to 0.73±0.28 (Iμ/l). Prolactin levels decreased significantly ($p<0.05$) from 0.53±0.07 to 2.31±0.35 (μg/ml) after ingestion of 48 mg/kg SHP. Administration of SHP had no significant effect on the testosterone of SD rats when compared with the control.

Effect of SHP on MDA level in testes.

MDA level was significantly ($p<0.05$) increased from 0.98±0.13 to 1.20±0.26 in the control untreated and 480 mg/kg SHP treated groups respectively..

Histopathological examinations

Histological examination of the testes (Plate1), showed the testes in the control with normal spermatozoa, but the treated group showed varying necrosis of the seminiferous tubules.

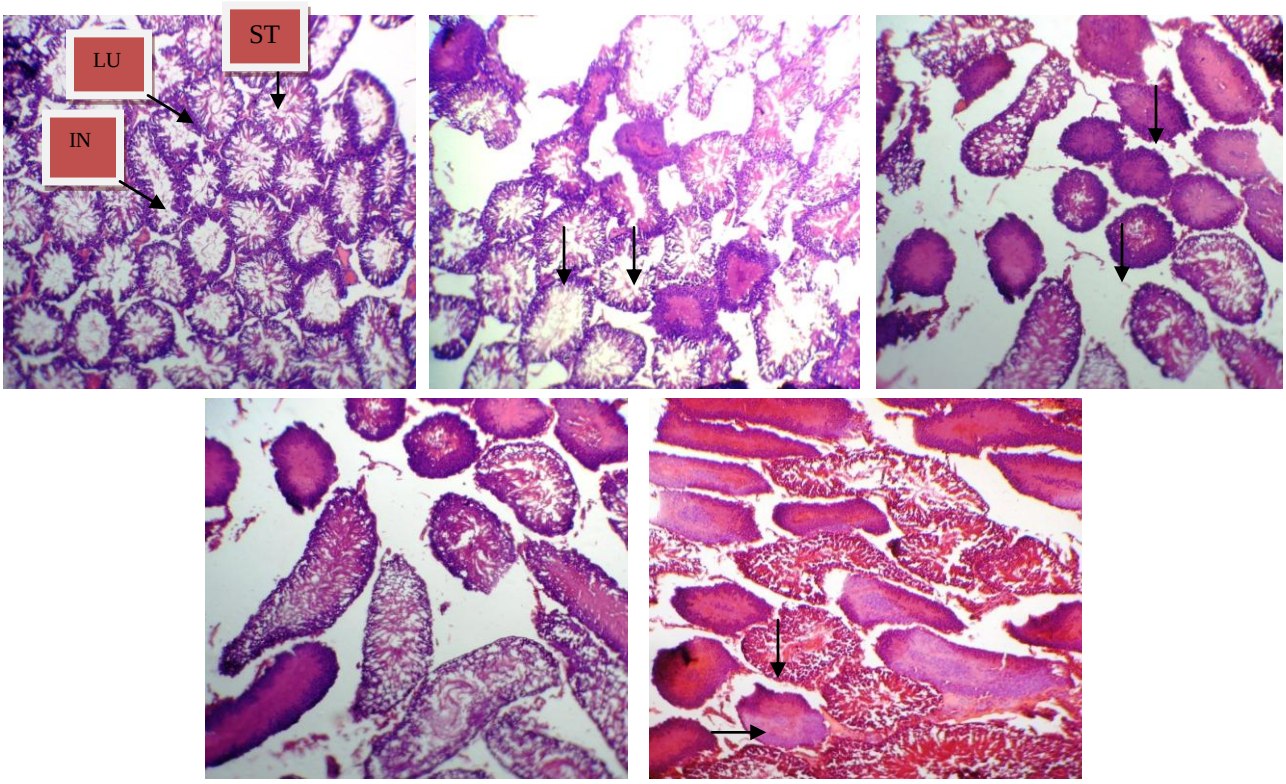

Fig. 1 Representative Photomicrograph ((H & E stain x 100) of the testis in male Sprague-Dawley rats after 90 days of oral administration of SHP. A. Control animal testis showing seminiferous tubules with numerous spermatozoa and germ cells. B. Test animal testis showing scanty focal infarction (coagulative necrosis) of seminiferous tubules (48mg/kg). C.Test animal testis showing mild focal infarction (coagulative necrosis) of seminiferous tubules(240mg/kg). D&E. Test animal testis showing marked focal infarction (coagulative necrosis) of seminiferous tubules (480mg/kg).

Table 1: Effect of SHP on Weigh gain, Feed Consumption, Percentage Weight Gain, Fluid Intake and Feed Efficiency.

	Study group (o-90days)				Recovery group			
	SHP treated group (mg/kg)				SHP treated group (mg/kg)			
	control	48	240	480	control	48	240	480
Treatment								
Total weight gained(g)	281.3	249	264	238	144.4	123.8	129.1	107.8
Testes organ weight(g)								
Absolute weight(g)	1.30±0.07	1.35±0.03	1.31±0.05	1.41±0.05	1.30±0.05	1.33±0.11	1.28±0.14	1.36±0.06
Relative weight (g)	0.54±0.03	0.45±0.02	0.50±0.02	0.50±0.01	0.49±0.01	0.53±0.04	0.46±0.05	0.48±0.02
Total feed consumed (g)	1016±67.57	1463±54.80**	1545±68.53**	1610±57.47**				
Percentage weight gain(g)								
30 days	32.5	27.64	27.68	18.43	52.79	47.92	45.79	37.85
60 days	45.73	39.74	36.35	33.85				
90 days	46.05	44.74	43.69	37.88				
Mean fluid intake (ml)	27.84±0.50	25.20±0.83**	25.99±0.57**	34.86±0.82				
Feed efficiency (%)	16±1.34	9.16±0.59	8.21±0.32	7.67±0.35				

Data are presented mean±SEM. Significant differences are marked**p<0.001,***p<0.0001.
Study group n=15, recovery groupn=5
Significantly different from control group, dingificantly different from 480mg/kg group

Table 2: Effect of SHP on hormones and Sperm Parameters.

	Study group (0-90days)				Recovery group			
	SHP treated group(mg/kg)				SHP treated group (mg/kg)			
Treatment								
Testosterone(ng/ml	2.29±0.63	2.31±0.35	2.21±0.46	0.09±0.26	3.22±0.40	1.56±0.15*	2.78±0.21	1.58±0.63*
Follicular stimulating hormone(FSH)IU/ml	0.82±0.45	0.88±0.58	1.24±0.93	0.68±0.41	0.42±0.07	0.34±0.05	0.34±0.02	0.28±0.03
Leutinizing hormone (LH)IU/ml	0.69±0.28	0.73±0.29	0.66±0.32	0.60±0.34	0.50±0.05	0.38±0.02	0.26±0.04*	0.20±0.03
Prolactin(ng/ml)	0.53±0.07	0.47±0.06	0.47±0.07	0.42±0.07	0.48±0.02	0.60±0.11	0.42±0.13	0.44±0.05
Sperm number								
Testicular sperm number(TSN)x10^6	63.53±15.5	63.87±10.77	65.93±11.24	65.25±16.75	66.60±6.52	73.40±10.22	64.80±9.83	77.60±7.13
Epidydimal sperm number(ESN)x10^6	64.13±1637	58.87±12.57	63.07±8.66	56.47±15.73	67.20±6.45	73.40±10.22	64.40±8.66	77.60±7.14
Debris(%)								
Testicular	19.10±5.78	25.85±3.64	49.80±5.66**	57.50±6.90**	14.20±1.91	17.80±1.50***	35.20±2.74	40.00±2.24***
Epidydimal	14.00±0.00	23.33±1.45*	54.00±4.00***	50.00±1.53***	14.00±2.46	30.00±5.24	58.00±3.74	70.00±5.70
Motility(%)								
Testicular	71.87±12.98	52.93±7352	34.33±5.95*	27.13±6.64*	76.50±7.32	88.12±3.00	71.00±5.00	74.13±5.16
Epidydimal	70.67±8.33	52.87±4.04	32.00±2.65**	22.00±4.73***	82.00±5.61	85.60±4.99	73.20±1.83	65.67±6.00
Sperm viability(%)								
Testicular	73.50±9.50	46.53±0.53*	28.87±2.38***	26.07±1.98***	87.10±1.86	80.00±1.52	65.70±2.27*	62.40±3.78**
Epidydimal	74.93±7.54	35.13±2.47***	15393±.0.13***	14.13±1.18***	80.80±4.42	72.00±6.33	72.00±6.33	47.00±5.19*
Abnormal sperm morphology(%)								
Testicular	15.67±7.67	14.33±6.84	11.00±4.04	13.00±6.51	9.00±1.00	8.00±1.23	8.00±1.23	6.00±1.01
Epidydimal	13.00±7.51	12.00±5.03	10.67±.3.71	13.33±7.33	5.00±0.00	10.00±0.00	6.00±1.00	9.00±1.00*

Data Are Presented as Mean ± SEM. Significant Differences are Marked *<p0.05, **p<0.0001,
Trudy Group n=15, Recovery Group n=5
Significantly Different from Control Group, Significantly Diffent from 480mg/kg group

DISCUSSION

Until recently, very few reports evaluate the commercially available herbal products. However, the effect of SHP on the male reproductive system has not been investigated despite its use in traditional herbal medicine as a fertility-enhancing agent.

Guidelines on toxicity testing place considerable emphasis on reporting changes in weight gain (OECD, 1998 & 2001). Body weight measurement provides a good indicator for the overall animal health status and may be relevant to the interpretation of reproductive effects. Generally, reduction in body weight and relative organ weights is a simple and sensitive index of toxicity after exposure to a potentially toxic substance (Teo et al., 2002; Kluwe, 1981). Lee et al. (2012) also stated that an increase or decrease in body weight, of an animal may indicate important physiological changes such as liver or hormonal changes or failure to absorb components such as proteins ,aminoacids and others.

Despite the popular scientific perception that xenobiotic compounds influence the body weight gain on prolonged ingestion,the total weight gain by the treated animals was higher and feed consumption of the treated group was significantly higher than that of the control. However, SHP tended to reduce the percentage weight gain during the study in the treated groups when compared with the control. The percentage body weight increase was found to be higher in the control group than in the SHP treated groups and was dose dependent. Because treatment with SHP did not affect the weight increase, it can be concluded that SHP did not produce any generalized toxicity. The feed efficiency is the

gram of body weight gain, per gram of food consumed over a period of time, the higher the value, the more efficiently the animal was able to utilize the food. Feed efficiency in the treated groups when compared with the control, was lower in the treated groups in a dose dependent manner. Although it had no effect on the appetite and overt general rat toxicity in this study, however, SHP may have reduced food conversion.

Although the non depreciation in the weight of the SHP treated animals, does not support overt toxicological involvement ,however the reduced percentage weight gain by the treated group and the reduced feed efficiency suggests a form of toxicity that manifested in form of metabolic assimilation of consumed food, or as the alteration of the metabolic processes of the treated animal (Cajuday and Poscidio, 2010). Though in one study (Awodele et al., 2012), *Moringa Oleifera* did not cause any percentage weight gain in the animals. The mechanism leading to reduced feed efficiency and percentage weight gain is not known.

Water is an essential nutrient to every life, since it is the most important nutrient for growth and development (Panagiotakos et al., 2003). Any factor influencing water intake will also affect feed consumption (Counotte, 2003). The fluid intake in the control did not differ from the other treated groups, except for the highest treated group where the water intake was significantly different from the control .Also weight of the testis in the SHP-treated groups did not decrease significantly when compared with the control group. The non significant reduction of testes weight is an indication that SHP did not alter the androgen status (Biswas *et al.*, 2001).

Many conditions or events associated with male infertility are inducers of oxidative stress, which leads to an increase in germ cell apoptosis and subsequent hypo-spermatogenesis, endocrine signaling, and germ cell apoptosis. Moreover, reactive oxygen species and oxidative damage of sperm bimolecular layer may contribute to male infertility by reducing sperm function (Atessahin *et al*., 2005). SHP did not affect the TSN significantly but induced marked reduction in epididymal sperm counts and percentage motility were significantly decreased. Some reports revealed that chemical ingestion, cause suppression of sexual behaviour of male rats (Hala *et al.*, 2010) and reductions in motility (Yousef *et al*., 2005). Prolactin in SHP fed groups was lower than control group. Prolactin can upregulate the synthesis of testosterone in vivo through cognate receptors on the Leydig cells of the testis (Purvis *et al*., 1979; Mathur *et al.*, 1975). In males with abnormal semen parameters, including sperm motility, sex hormones are evaluated to rule out testicular causes and aid in the management. Low levels of FSH suggest a hypothalamo-pituitary axis problem and high levels of FSH signify testicular failure. SHP may be acting on the hypothalamo-pituitary axis since there was a reduction of FSH.

In the present study, exposure of rats to SHP did not decrease testicular sperm number, but decreased epidydimal sperm number, sperm motility and viability. SHP also did not increase sperm abnormalities. SHP ingestion did not impair spermato-genesis within the investigation period but adversely impacted the spermatozoa store in epidydimal compartment, reduced the motility and elevated spermatozoa abnormalities. These observations in the SHP treated rats might be the outcome of altered and hostile internal milieu of epididymis caused by reactive oxygen species since high dose of SHP led to significant increase in the MDA, an index of lipid peroxidation, in testes and spermatozoa of rats in the present study. Increased sperm membrane lipid peroxidation has been shown to impede sperm progress motility and increase percentage total sperm abnormalities as well as cause a dramatic loss in the fertilizing potential of sperm (El-Demerdash *et al*.,2004).

Apart from the production of spermatozoa, testis is involved in the production of hormones that are required for various functions in the body, including maintenance of secondary sexual functions, and feedback on the hypothalamus and the pituitary to control the secretion of the gonadotropins (Saradha & Mathur, 2006). The interstitial Leydig cells in the testis are almost exclusively responsible for the biosynthesis and secretion of testosterone which is the male primary steroid hormone. The secretion of testosterone is dependent upon the secretion of LH by the pituitary gland (Uzun *et al*., 2009). It promotes differentiation of spermatozoa during spermatogenesis. Besides, it has many important roles in the male reproductive functions such as inhibition of gonadotropin releasing hormone (GnRH) and LH secretion, induction and maintenance of the differentiation of male accessory reproductive organs (Dohle *et al.*, 2003). So, any change in the level of testosterone would have direct effects on spermatogenesis, including a decrease in sperm counts (McLachlan *et al.*, 1996). Since reduced epididymal sperm count correlates with suppressed level of testosterone in the circulation (Mali *et al*., 2002) and there was no significant reduction testicular sperm count, we may conclude that SHP did not affect spermatogenesis.

Rao *et al.*, (1989) reported declined sperm motility, resulting in decreased fertility. In another study sluggishly motile or immotile spermatozoa were found to be unable to penetrate the cervical mucus and to fertilize the ova (Sharma *et al.*, 1999). The decrease in sperm motility might be caused by a decrease in ATPase activity which might be linked to defects in the ultrastructure of sperm (Thangaraj *et al.*, 2003). The morphology and functional integrity of accessory sex tissues are dependent on androgens (Dohle *et al.*, 2003).

These results suggest that SHP beyond inducing alterations in testis histology also caused a reduction in sperm motility and increase in percentage sperm debris. Sections taken through the testes of control animals demonstrated normal histology. In contrast, sections taken through the testes of low, medium and high dose-receiving groups showed coagulative necrotic lesions in seminiferous tubules and also marked focal infarction (coagulative necrosis) of testicular seminiferous tubules, disorganized seminiferous tubule and widened spaces between neighboring tubules. The effect was more pronounced in the higher dose treated rats than in the lower dose treated rats. Based on the results of our study, we conclude that SHP acted as an

antifertility agent instead of a fertility booster. This is demonstrated by the decrease in the fertility parameters (motility, viability, debris), in addition histological results of the testes of the treated rats showed coagulative necrosis of the seminiferous tubules.

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