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EFFECT OF NIFEDIPINE ON FERTILITY OF MALE LESSER BANDICOOT RAT (*Bandicota bengalensis*)

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ABSTRACT

Current study was designed to evaluate the antifertility effects of nifedipine, a pharmaceutical medicament for high blood pressure. Mature and healthy male lesser Bandicoot rats, *Bandicota bengalensis* Gray and Hardwicke, were fed on bait (cracked wheat, powdered sugar, groundnut oil 98:2:2) containing 0.1 and 0.05% nifedipine for 12 days in no-choice feeding test to record antifertility effects of nifedipine. The factors studied were the effect on weight of reproductive organs, sperm parameters in cauda epididymal fluid, breeding performance and histo-morphology of testis. Nifedipine treatment significantly *(P <0.05)* reduced the weight of seminal vesicles and prostate gland in treated groups as compared to untreated group. Sperm motility, live sperm count, sperm density and sperm morphology differed significantly between untreated and treated groups. The breeding performance of treated male rats was found reduced by 40-60% as compared to untreated ones. A significant effect of nifedipine treatment was observed on the number of germinal cells in seminiferous tubules. The study indicates the effect of nifedipine on fertility of male rats.

Key words: Antifertility effect, *Bandicota bengalensis*, histo-morphology, nifedipine, sperm parameters

INTRODUCTION

Nifedipine belongs to a class of drugs known as calcium channel blockers and is a high blood pressure medication taken as a daily pill. Dr. Susan Benoff was the first to recognize contraceptive effect of nifedipine in 1992 when a couple sought fertility treatment after multiple rounds of failed *in vitro* fertilization. The husband was taking nifedipine as a high blood pressure medication. Tests showed low levels of mannose lectin and higher than normal amounts of cholesterol on his sperm cell membranes (Benoff, 1999). When the husband changed to a different high blood pressure medication, the sperm membrane cholesterol level dropped and the couple was able to conceive within a few months (Hershlag *et al*., 1995). Based on *in vitro* studies, Kanwar *et al.* (1993) suggested the potential of calcium channel blocking agents in designing of male contraceptives. Till then only a few *in vitro* and *in vivo* studies have been conducted on nifedipine with regard to its antifertility effects (Iranloye *et al.*, 2009; Morakinyo *et al.*, 2009; Waghmare *et al*., 2011).

The lesser Bandicoot rat, *Bandicota bengalensis* Gray and Hardwicke is a predominant rodent pest species inhabiting irrigated crop fields in India (Parshad, 1999; Singla and Parshad, 2010) and responsible for causing heavy losses to field crops (Singla and Babbar, 2010, 2015). It is also held

responsible for transmitting various diseases of man and livestock (Singla *et al.*, 2008, 2013b, 2016). The economic losses and health problems associated with this pest emphasize the need to develop techniques for its management. Presently, the main approach being followed to tackle rodent problems all over the world is to kill them with rodenticides. However, the excessive use of rodenticides can result in environmental hazards due to direct and indirect poisoning of non-target organisms (Mineau *et al.*, 2004; Brakes and Smith, 2005). Reports have reveal the development of resistance to rodenticides and the associated impact on pest-control programmes world-wide (Misenheimer *et al.*, 1994; Cowan *et al.*, 1995; Pelz *et al.*, 2005). After a successful control operation with rodenticides, rodents rapidly rebuild their population (Shilova and Tchabovsky, 2009) owing to their high reproduction rate. So, the challenge is to develop strategies which can reduce post-control reproductive output of rodents as it may be more practicable to prevent animals from being born than to reduce their numbers after they are partially or fully grown and established in a secure environment (Balser, 1964).

Many compounds have so far been tested for their toxic and antifertility effects in rats and mice (Cooper *et al*., 1997; Singla and Parshad, 2001; Ajamorooz *et al*., 2007; Singla and Pashad, 2009; Singla *et al*., 2013a; Singla and Challana, 2014, 2015), but not a single compound is used practically to control rodent populations due to one or more of their drawbacks. The association between male factor infertility and exposure to pharmaceutical compounds has only recently been explored and is a subject sure to generate more research and discussion in the years to come (Brezina *et al*., 2012). Based on our present knowledge of antifertility agents in vertebrate pest control, it appears that we still need to evaluate potential antifertility agents along with the techniques of their application. The present study was hence undertaken to determine the antifertility effects of nifedipine in male rats, *B. bengalensis*.

MATERIALS AND METHODS

Collection and maintenance of animals

Mature and healthy male lesser bandicoot rats, *B. bengalensis*, were live-trapped with single and multi-catch rat traps from crop fields and premises in and around Ludhiana (India). In the laboratory, rats were acclimated individually in cages (36 cm × 23 cm × 23 cm) for 10-15 days before the commencement of experiment with food and water provided *ad libitum*. Food consisted of a loose mixture of cracked wheat, powdered sugar and groundnut oil (WSO bait) in a ratio of 96:2:2 (Singla and Kaur 2016). Animals were maintained as per the guidelines of Institutional Animal Ethics Committee (sanction no. 1721-43 dated 28.06.2011).

Treatments

Nifedipine (molecular formula $C_{17}H_{18}N_2O_6$, molecular weight 346.3) was procured from MP Biomedicals, USA. Animals were weighed (279.44 ± 13.62 g) and divided into three groups of 6 each. There was no significant difference in body weight of rats in treated and untreated groups. Rats in groups I and II were fed on WSO bait containing 0.1 and 0.05% nifedipine, respectively, for 12 days in no-choice feeding test while rats in group III were fed on plain WSO bait and kept as untreated control. Food was kept in food bowls and water was provided *ad libitum*.

Before and after the treatment, all the rats were fed on plain WSO bait. Food consumption was recorded after every 24 h and every time feed was replenished to the original 30 g. Before weighing, the feed of all the treated and untreated rats was cleared of fecal pellets and dried. Mean daily consumption of feed {g 100 g^{-1} body weight (b.w.)} was calculated separately for each group of rats during pre-treatment, treatment and post-treatment periods based on their body weight. Rats were also observed for mortality and other clinical symptoms. From the total amount of treated food

consumed during the treatment period, the mean total and mean daily dose (mg kg^{-1} b.w.) of nifedipine ingested by each group of rats was calculated. The percent acceptance of treated food over plain bait consumed before treatment was determined as per the formula given below:

$$\text{Percent acceptance} = \frac{\text{Consumption of food during treatment} \times 100}{\text{Consumption of food during pre-treatment}}$$

Antifertility effects

After the termination of treatment, all the treated and untreated male rats were paired with untreated cyclic female rats in 1:1 ratio. Food and water were provided *ad libitum.* After 15 days of pairing, female rats were separated from male partners and observed for pregnancy and delivery of pups.

After 30 days of treatment withdrawal, male rats were weighed and sacrificed to record antifertility effects of nifedipine. Their reproductive organs and accessory sex glands, such as testis, epididymis, seminal vesicles and prostate gland were dissected out to determine their weights (g 100 g^{-1} b.w.). The effect of nifedipine on sperm parameters, such as sperm motility (%), live sperm count (%), sperm density (millions mL^{-1}) and sperm morphology (%) in the cauda epididymal fluid, was determined as per the methods described by Salisbury *et al.* (1978). Sperm motility was assessed immediately after autopsy and expressed as percent motility. The live sperm count was determined using 0.2% eosin stain, sperm density by using hemocytometer and sperm morphology from a count of 300 spermatozoa in Giemsa-stained smears of cauda epididymal fluid per animal.

A piece of testicular tissue was fixed for 48 h in aqueous Bouin's fluid. Then the tissue was processed and embedded in paraffin wax (Humason, 1979). The sections of 5 µm thickness were cut in a microtome and, after dewaxing, the sections were processed and stained with hematoxylin and eosin (H & E). Stained sections of testis from each rat were studied under a light microscope for the development of cellular associations in seminiferous epithelial cycle (SEC) in seminiferous tubules (Perey *et al.*, 1961). The diameter of almost round seminiferous tubules and thickness of germinal epithelium were measured by examining 25 tubules $animal^{-1}$. Measurements were made using an ocular micrometer calibrated with a stage micrometer. The effect of nifedipine treatment on histomorphology of testis was assessed quantitatively by counting the number of nuclei of spermatogonia, leptotene, zygotene, pachytene and diplotene spermatocytes, round spermatids, elongating spermatids, elongated spermatids, spermatozoa and Sertoli cells in almost round cross-sections of 25 seminiferous tubular sections $animal^{-1}$. Crude spermatogenic cell count of round and almost round nuclei was corrected for differences in their nuclear diameter to obtain true count of cells using Abercrombie's formula (Abercrombie, 1946; Rana and Bilaspuri, 1994) as given below:

$$\text{True count} = \text{Crude count} \times \text{Correction factor}$$

Where,

$$\text{Correction factor} = \frac{\text{Section thickness}}{\text{Section thickness} + \sqrt{(\text{average diameter}/2)^2 - (\text{average diameter}/4)^2}}$$

Statistical analyses

Data was expressed as mean ± SE and analyzed using analysis of variance and Student's t-test. P-value <0.05 was considered significant. The values wherever necessary were subject to arc sign transformation prior to analysis.

RESULTS AND DISCUSSION

Bait acceptance and active ingredient ingested

Results revealed no significant differences between treated and untreated groups of rats for mean daily consumption of food during treatment period (Table 1). There also was no significant difference

Table 1: Acceptance of WSO bait containing nifedipine and amount of nifedipine ingested by *B. bengalensis*

Treatment (n = 6 each)	Mean daily consumption (g 100 g^{-1} b.w.)			Acceptance of treated bait (%)	Dose of nifedipine ingested (mg kg^{-1} b.w.)
	Pre-treatment	Treatment	Post-treatment		
0.1%	7.98±0.17	4.81±0.40	5.46±0.10*	60.20±4.69	576.53±47.82
0.05%	6.71±0.11	4.50±0.51	4.76±0.12*	67.25±3.75	269.90±12.57
0%	6.99±0.19	5.45±0.19	5.47±0.22	-	-

Values are mean ± SE; n = number of animals;
*Significant difference between pre- and post-treatment bait consumption at P <0.05

in food consumption before and during the treatment by same group of rats. The acceptance of bait containing nifedipine over plain bait consumed by same group of rats during pre-treatment period was 60.20 and 67.25% by rats treated with 0.1 and 0.05% nifedipine, respectively. Significant (*P* <0.05) effect of treatment was observed on post-treatment consumption of food by treated groups of rats. All rats behaved normally and were active throughout the period of experimentation. No mortality of rats was observed during the experiment. Feeding of bait containing 0.1 and 0.05% nifedipine resulted in the ingestion of total 576.53±47.82 and 269.90±12.57 mg kg^{-1} b.w. of nifedipine, respectively, in 12 days and 48.05 ± 3.98 and 22.49 ± 1.05 mg kg^{-1} b.w. of nifedipine daily. The LD_{50} of nifedipine in rats has been reported to be 1022 mg kg^{-1} b.w. (Lewis 2004). The doses of nifedipine ingested by rats in present study were lower than LD_{50} dose.

Effect on reproductive performance

Breeding of treated and untreated male rats with untreated cyclic female rats after 15 days of treatment withdrawal revealed 83.3% (5/6) breeding in untreated male rats. The number of pups delivered by different females varied from 3 to 5 with average of 4.20±0.33. In male rats treated with 0.1% nifedipine, breeding was 33.3% (2/6). Only one pup was delivered by each of the two females. In male rats treated with 0.05% nifedipine, breeding was 50% (3/6). The number of pups delivered by three females varied from 1 to 2 with average of 1.33±0.27. Thus, there were 60 and 40% reductions in breeding performance in rat groups treated with 0.1 and 0.05% nifedipine, respectively, along with reduction in number of pups delivered from that of untreated group.

Effect on organ weights

No significant effect of feeding WSO bait containing 0.1 and 0.05% nifedipine for 12 days in no-choice feeding test was observed on mean weights of testis and epididymis in rats autopsied 30 days after treatment withdrawal. The weights of seminal vesicles and prostate gland were, however, significantly low in both the treated groups (Table 2). The differences in weights of these organs between treated groups were non-significant. There were no dose dependent reductions in weights. The reduction in weights of testis, epididymis, seminal vesicles and prostate gland in treated groups as compared to untreated group were 20.4-31.8, 33.3-40.0, 47.2-59.7 and 32.0-68.0%, respectively (Fig. 1). Mean body weights of treated groups of rats did not differ significantly from that of untreated group 30 days after treatment withdrawal. Significant reduction in weights of testis and epididymis has been reported in male rats orally treated with 0.57 mg nifedipine kg^{-1} b.w. for 30 days (Iranloye *et al*., 2009; Moralinyo *et al.*, 2009). Rats were sacrificed after 30 days of treatment withdrawal to record the effect of nifedipine up to at least two spermatogenic cycles.

Table 2: Effect of nifedipine treatment on weights of reproductive organs of *B. bengalensis*

Treatment (n = 6 each)	Body weight (g)	Weights (g 100g^{-1} b.w.)			
		Testis	Epididymis	Seminal vesicles	Prostate gland
0.1%	281.67±15.75	0.30±0.02	0.09±0.01	0.38±0.05 ^{a}	0.17±0.02 ^{a}
0.05%	230.00±15.90	0.35±0.05	0.10±0.01	0.29±0.09 ^{a}	0.08±0.02 ^{a}
0%	318.33±24.77	0.44±0.01	0.15±0.01	0.72±0.05 ^{b}	0.25±0.02 ^{b}

Values are mean ± SE; n = number of animals; The values with different superscripts in a column differ significantly at P < 0.05

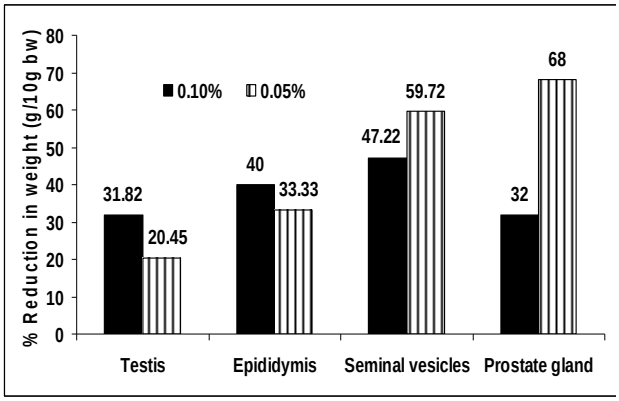

Fig. 1: Percent reduction in weights of reproductive organs in treated groups of rats from that of untreated group of rats

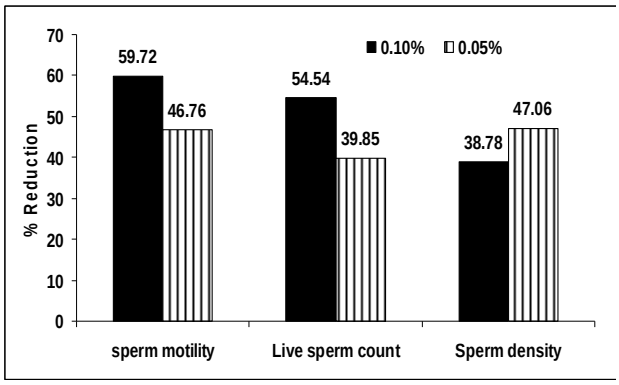

Fig 2. Percent reduction in sperm parameters in treated groups of rats from that of untreated group of rats

Effect on sperm parameters

A significant effect ($P < 0.05$) of nifedipine treatment was observed on sperm motility, live sperm count, sperm density and sperm morphology in the cauda epididymal fluid of treated rats (Table 3). No dose-dependent effect of treatment was observed on sperm parameters in different rat groups. A significant difference in values of these parameters was observed between groups of treated and untreated rats, however, similar differences between treated groups were non-significant. The reductions in sperm motility, live sperm count and sperm density in treated groups of rats compared to untreated group were 46.76 to 59.72, 39.85 to 54.54 and 38.78 to 47.06%, respectively, after 30 days of treatment withdrawal (Fig. 2). The effect of nifedipine treatment on sperm morphology was in the form of abnormality in sperm head. shape and coiling of midpiece and tail (Fig. 3). The average sperm abnormality was up to 43.38 and 36.92%, respectively, in rats treated with 0.1 and 0.05% nifedipine as compared to 19.15% abnormality observed in untreated rats. Most of the sperm heads in cauda epididymal fluid of treated rats were without acrosomal cap. The calcium channel blockers have been shown to produce a dose dependent reduction in sperm motility and viability in *in vitro* studies (Aaberg *et al*., 1989). *In vitro* studies with analogues of nifedipine have revealed their action by altering the cell metabolism thereby directly affecting the sperm motility which is responsible for fertility (Waghmare *et al.,* 2011). Treatment of male rats with 0.57 mg nifedipine kg^{-1} b.w. for 30 days significantly decreased sperm count and motility in treated rats (Iranloye *et al.,* 2009; Moralinyo *et al*., 2009). The *in vitro* studies by Kanwar *et al.* (1993) revealed that the addition of 0.1-100 µL nifedipine in epididymal fluid resulted in a significant non-competitive inhibition in Ca^{2+} uptake. The activity of Ca^{2+}-dependent ATPase enzyme was also decreased. Motility of spermatozoa was significantly arrested following the incubation with different doses of drug at 37^0C for different durations. Scanning electron microscopic studies revealed disruptive changes in the head as well as tail region and coiling of spermatozoa after nifedipine treatment.

Table 3: Effect of nifedipine treatment on sperm parameters of *B. bengalensis*

Treatment (n = 6 each)	Body weight (g)	Sperm motility (%)	Live sperm count (%)	Sperm density (x 10^6 mL^{-1})	Sperm abnormality (%)
0.1%	281.67±15.75	29.00±8.03 [a]	35.08±8.58 [a]	258.67±40.33 [a]	43.38±1.24 [a]
0.05%	230.00±15.90	38.33±11.88 [a]	46.42±12.13 [a]	223.67±82.20 [a]	36.92±5.32 [a]
0%	318.33±24.77	072.00±1.90 [b]	77.17±2.30 [b]	422.50±11.16 [b]	19.15±0.54 [b]

The values are mean ± SE; n = number of animals; The values with different superscripts in a column differ significantly from each other at $P < 0.05$

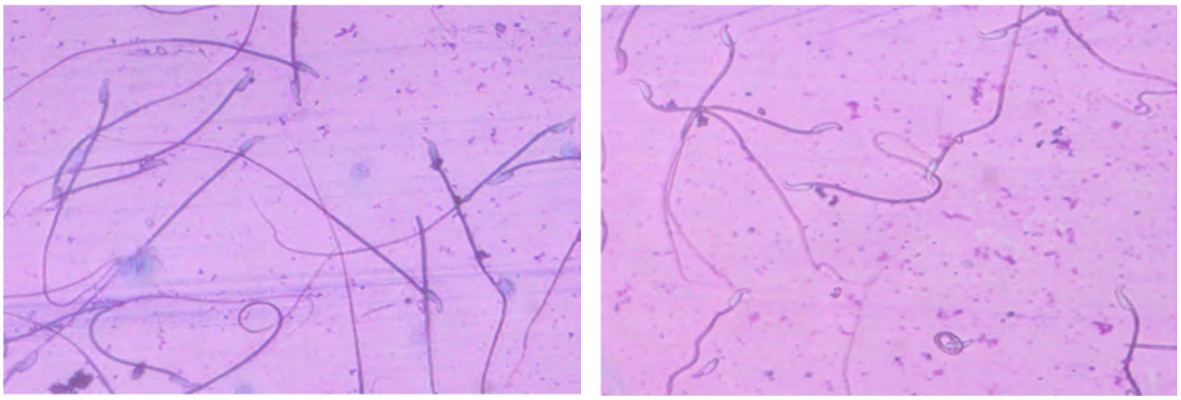

Fig. 3: Sperms abnormality observed in rats treated with nifedipine at 400 x magnification: Most of the sperm heads without acrosomal cap (left), Sperms with coiled midpiece and tail (right)

Sperm functions such as count and motility are the key indices of male fertility because these are prime markers in testicular spermatogenesis and epididymal maturation (Morakinyo *et al.*, 2009). The reduction of these parameters by nifedipine treatment in our studies is in agreement with the findings of Juneja *et al.* (1990) and Almeida *et al.* (2000) who reported decrease in sperm count and motility with other calcium channel blockers. Calcium channel blockers may inhibit the entry of calcium into sperm membranes, thereby affect the flagella motility of sperm cell. Calcium channel blockers have also been proposed to mimic gossypol, a phenolic aldehyde extracted from cotton seeds in its effect on sperm function (Morakinyo *et al*., 2009; Singla and Meenu, 2007). Because of the lipophilic nature of gossypol, it interacts directly with the phospholipid bilayer of biological membranes altering their structure, electrostatic charges and transmembrane ion fluxes. When studied in animal models, gossypol reportedly inhibited calcium ion uptake, Ca^{2+}, Mg^{2+}, ATPase and Ca^{2+}-ATPase activity (Kanwar *et al*., 1993). Nifedipine also affects changes in sperm membrane structure and functions similar to those of gossypol (Benoff *et al.*, 1994; Goodwin *et al.*, 1997). These changes include blocking of mannose lectin, an important protein on sperm membrane critical for binding with an egg's zona pellucida. Nifedipine treatment may physically prevent mannose lectins from moving to the surface of cell membrane thereby leading to the stiffening of membrane with excess of cholesterol on sperm head, thus affecting the normal movement of sperm (Benoff *et al.*, 1994, Goodwin *et al.*, 1997; Benoff *et al.*, 1998).

Most of the sperm heads in cauda epididymal fluid of treated rats were found without acrosomal cap. The acrosome reaction is a complex calcium dependent process (Fraser, 1993). Premature spontaneous acrosome reaction prior to reaching the oocyte may lead to the early sperm cell death. It has been demonstrated that nifedipine, a calcium channel blocker, has the ability to block acrosome reaction (Fraser and Mclntyre, 1989). A group of men taking nifedipine for hypertension were found to have low rates of acrosome reaction during capacitating conditions. *In vitro* introduction of nifedipine in the medium of sperm from normal donors also confirmed these results Benoff *et al*., 1994). Following cessation of drug, the acrosome reaction status returned to normal and subsequently pregnancy was achieved (Hershlag *et al*., 1995).

Effect on histomorphology of testis

At light microscopy level, all the stages of SEC (i.e. from stages 1 to IV) were fully matured in the cross-sections of testis stained with H&E in both treated (Fig. 4) and untreated rat groups (Fig. 5). No significant difference was observed in the diameter of seminiferous tubules and thickness of germinal epithelium among treated and untreated groups (Table 4). Mean diameter of seminiferous tubules varied from 0.23 to 0.24 mm in treated rats as compared to 0.26 mm in untreated rats. The thickness of germinal epithelium varied from 0.11 to 0.12 mm in both treated and untreated groups.

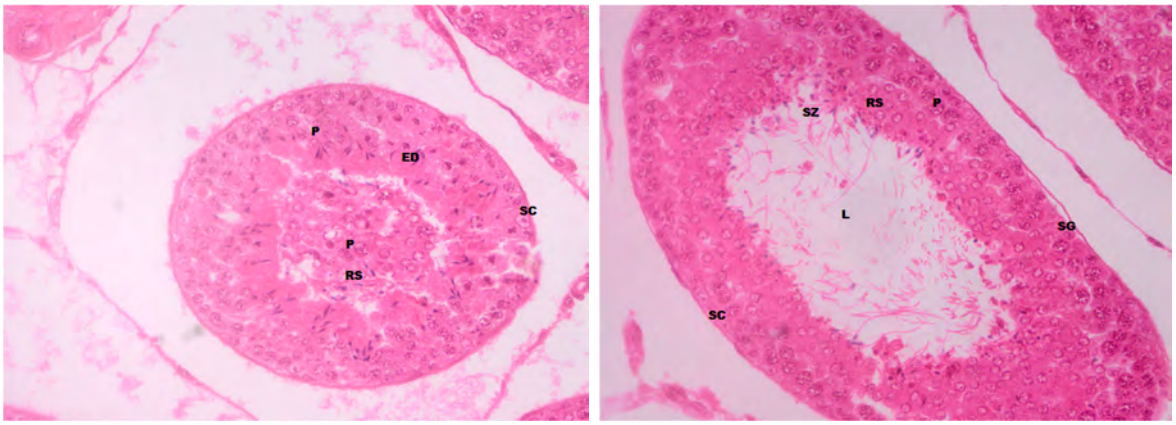

Fig. 4: HE stained section of seminiferous tubule in testis of treated rat at 400x magnification showing normal cell associations but reduced number of germ cells: Seminiferous tubule in stage IV-VII of the seminiferous epithelial cycle with some cells lying in lumen (left); Seminiferous tubule in stage VI-VIII of the seminiferous epithelial cycle with degenerating spermatozoa in lumen; SG, Spermatogonium; SC, Sertoli cell; P, Pachytene spermatocyte; RS, Round spermatid; ED, Elongating spermatid; SZ; Spermatozoa; L, Lumen (right)

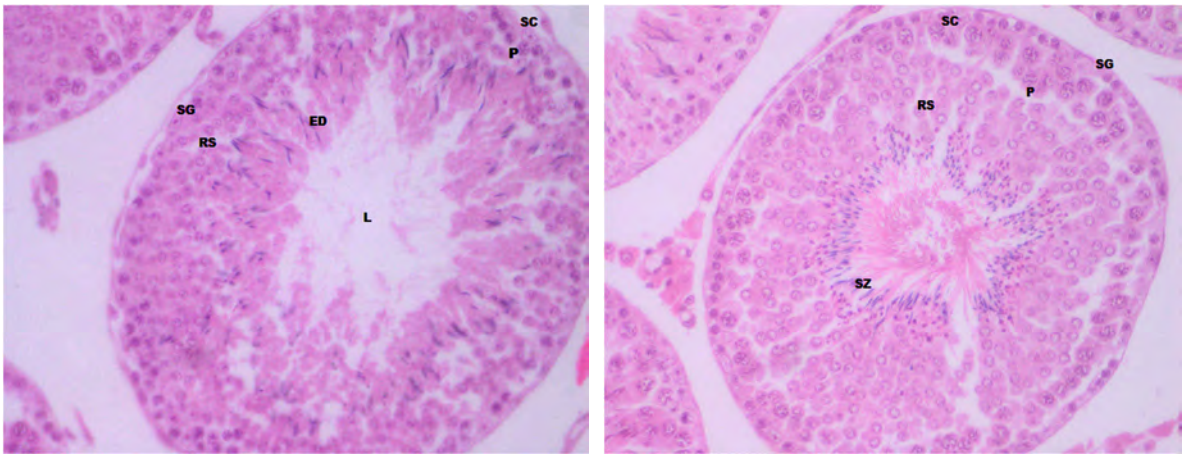

Fig. 5: HE stained section of seminiferous tubule in testis of untreated rat at 400x magnification showing normal cell associations: Seminiferous tubule in stages IX-XII of the seminiferous epithelial cycle (left); Seminiferous tubule in stage IV-VII of seminiferous epithelial cycle; SG, Spermatogonium; SC, Sertoli cell; Z, Zygotene spermatocyte; P, Pachytene spermatocyte; RS, Round spermatid; ED, Elongated spermatid; SZ; Spermatozoa; L, Lumen (right)

Table 4: Effect of nifedipine treatment on histomorphology of testis of *B. bengalensis*

Treatment (n = 6 each)	STD (mm)	TGE (mm)	SG	L	Z	P	D	RS	EL	ED	SZ	SC	Total
0.1%	0.24± 0.00	0.12± 0.00	6.20± 0.14 [a]	3.00± 2.12 [a]	4.25± 0.18 [a]	37.18± 3.76 [a]	23.75± 0.71 [a]	57.24± 7.69 [a]	51.75± 0.18 [a]	39.18± 5.43 [a]	35.47± 2.63 [a]	9.00± 0.00 [a]	267.03± 14.11 [a]
0.05%	0.23± 0.01	0.11± 0.01	5.87± 0.14 [a]	5.17± 2.36 [a]	6.51± 0.49 [a]	36.70± 2.69 [a]	25.95± 0.50 [a]	66.09± 3.22 [a]	61.52± 4.93 [a]	65.17± 1.21 [a]	42.98± 2.40 [a]	8.70± 0.12 [a]	324.66± 7.78 [a]
0%	0.26± 0.01	0.11± 0.00	11.45± 0.03 [b]	10.50± 0.35 [b]	29.50± 2.47 [b]	44.10± 1.34 [b]	50.00± 2.12 [b]	78.50± 2.95 [b]	82.75± 1.59 [b]	90.50± 0.35 [b]	98.50± 0.71 [b]	14.55± 0.11 [b]	510.35± 5.25 [b]

Values are mean ± SE; n = number of animals; The values with different superscripts in a column differ significantly at $P < 0.05$: STD, Seminiferous tubule diameter; TGE, Thickness of germinal epithelium; SG, Spermatogonia; L, Leptotene spermatocytes; Z, Zygotene spermatocytes; P, Pachytene spermatocytes; D = Diplotene spermatocytes; RS = Round spermatids; EL = Elongating spermatids; ED = Elongated spermatids; SZ = Spermatozoa; SC = Sertoli cells

A decreased number of different germinal cells were found in the seminiferous tubules of treated rat groups as compared to untreated group. The total average count of cells per seminiferous tubule in group of rats treated with 0.1% nifedipine was least (267.03 ± 14.11) followed by that in group treated with 0.05% (324.66 ± 7.78) and untreated group of rats (510.35 ± 5.25) (Table 4). The average number of Sertoli cells varied from 8.7 to 9.0 in treated groups as against 14.55 in untreated group of rats. The values of different germinal cells were significantly low in treated groups than untreated group of rats. The difference in number of different cells between the two treated groups was, however, found to be non-significant. Similar to present studies, Iranloye *et al.* (2009) also observed biologically no meaningful change in the histological section of testis of rats treated with nifedipine as compared to control tissue. Morakinyo *et al.* (2009) reported no effect of calcium channel blockers on reproductive hormones such as LH, FSH and testosterone. Therefore, it appears that the primary site of action of these chemicals is not the hypothalamic-pituitary-testicular axis, but the effect may directly be on the reproductive tissues and cells (Goodwin *et al.,* 1997). Morakinyo *et al.* (2009) observed reversal in antifertility effects of nifedipine in male rats after 30 days of withdrawal of treatment (0.57 mg kg^{-1} b.w. day^{-1} for 30 days). During present studies, rats of groups treated with 0.05 and 0.1% nifedipine ingested daily on an average 22.49 and 48.05 mg nifedipine kg^{-1} b.w., respectively, which is a high dose than that administered by Morakinyo *et al.* (2009). In present study, the antifertility effects of nifedipine were observed after 30 days of treatment withdrawal. The studies, however, could not take into account the reversibility in antifertility effects of nifedipine after longer periods of treatment withdrawal. The present study thus records the antifertility effects of nifedipine in male rat, *B. bengalensis,* when fed in bait form on very high doses.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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