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Long term exposure with indoxacarb in WLH cockerels produces reproductive toxicity and its amelioration by rhizome powder of Shati (*Hedychium spicatum*)

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Abstract

The toxic effects of indoxacarb on the reproductive organ of cockerel and protective effect of Gandhpalasi (*Hedychium spicatum*) were evaluated in this study. The dose of indoxacarb was selected on the basis of LD_{50} in cockerels (250mg/kg). The experimental birds were kept in deep litter system of housing and maintained on grower ration. Birds were sacrificedafter completion of 16 weeks feeding trial. The semen were collected for estimation of mass motility, sperm count and % dead sperm. The antioxidant biomarkers in testis were also estimated. Finally the microscopic and ultrastructure of the testes were seen comparatively in both indoxacarb intoxicated and HSRP treated cockerels. The significant ($P<0.05$) reduction in sperm count and mass motility in indoxacarb group II and simultaneous treatment with HSRP in group VI and VII showed significant ($P<0.05$) improvement which was at par with the control. The % dead sperm were increased significantly ($P<0.05$) in indoxacarb treated group II whereas in HSRP treated group VI and VII showed significant decline in dose dependent manner. The antioxidant biomarker were significantly ($P<0.05$) changes by indoxacarb intoxication but treatment HSRP produced significant ($P<0.05$) amelioration. At the time of microscopic and ultrastructure examination of testes showed changes in indoxacarb treated group II and in HSRP treated group VI and VII showed mild to moderate changes. It is concluded from the present study that the HSRP produced protective effect in indoxacarb induced reproductive toxicity and its amelioration by HSRP treatment.

Keywords: Indoxacarb, Cockerel, Gandhpalaasi (*Hedychium spicatum*), Reproductive toxicity

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Introduction

Reproduction is important physiological phenomenon which is controlled by the different hormones. Indiscriminate use of pesticides and chemicals in modern life and in agricultural practices leads to disruption of normal endocrine physiology and affects the reproductive cycle in all animals and birds. A large number of chemicals that have been released into the environment are known to interfere with the endocrine system. Sexual development during the prenatal and neonatal period is under hormonal control and is, therefore, sensitive to exogenous substances with an endocrine effect. Keeping in mind this experimental work was done.

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Indoxacarb is a new oxidiazine group of pesticides used as acericides by inhibiting the sodium channels [1]. It produces toxicity by generating the reactive oxygen species (ROS) in testis [2, 3]. Indoxacarb produces toxicities in rat by decline in blood level of FSH and progesterone whereas testosterone levels were increased [4].The *Hedychium spicatum* is known as Shati and grown around the Himalayan region of Uttrakhand, Assam and Arunachal Pradesh. The plant contains glycosides, alkaloids, falavonoids, phenolics and other phytoconstituents[5] and has potent antioxidant capacity [6], have ameliorating potential in indoxacarb treated cockerels [7].

Materials and Methods

Plant materials

The rhizome of *Hedychium spicatum* L. was collected from the Medicinal Research Development Center, G. B. Pant University of Agriculture & Technology, Pantnagar. The rhizome was gathered from the plants, chopped like slice, shade dried for 15-20 days. Later the chopped rhizomes were placed in incubator at 37°C for 2-4 h and grounded in electric grinder to fine homogenous powder to get rhizome powders of *Hedychium spicatum* (HSRP). The powder was stored in sealed plastic container in dry place at room temperature till further use. The powder was used to feed the cockerels under study with the steamed standard computed ration.

Experimental Animals

Forty nine white leghorn cockerels, 6 to 8 weeks old, weighing 300 to 400 g were procured from Instructional Poultry Farm, G. B. Pant University of Agriculture & Technology, Pantnagar and maintained under deep liter system following standard management practices. Experimental birds were randomly divided into seven groups of 7 birds each and were kept for 15 day for adaptation. Feed and drinking (tap) water were given *ad libitum* during the study. Present study was conducted after approval by the Institutional Ethical Committee (IAEC) vides approval number IAEC/VPT/CVASc/193 dated. 27. 02. 2015. Indoxacarb (Avaunt 15.8 % EC) used in the study were purchased from local market.

Chronic oxidative stress was induced in WLH cockerels by indoxacarb @ 25mg/kg body weight. The dose of indoxacarb was selected on the basis of LD_{50} in cockerels (250mg/kg). The experimental plan in tabulated form is given as under

Table 1: Experimental plan

Group	No. of birds	Treatment
Group I (Control)	7	Standard ration *ad lib*
Group II	7	Indoxacarb (250 ppm) in feed
Group III	7	Silymarin (250 ppm) in feed
Group IV	7	Indoxacarb (250 ppm) + Silymarin (250 ppm) in feed
Group V	7	*Hedychium spicatum* rhizome powder-(HSRP) @ 4000ppm) in feed
Group VI	7	HSRP@ 2000ppm+indoxacarb@250ppm in feed
Group VII	7	HSRP@ 4000ppm+indoxacarb@250ppm in feed

Collection of semen

Semen was collected from the cockerels sacrificed after 16 weeks study. The right and left vas deferens were surgically excised from their point of entry at the cloacae to the inferior edge of kidneys. The contents of the vas deferens were collected in a test tube with maintaining the temperature at 38-40°C with by applying insulated jacket.

Mass motility of spermatozoa in neat semen

Mass motility in neat semen was estimated by the method[8]. A drop of semen was taken with the help of tips on a cleaned glass slide and covered with a glass cover slip and kept under microscope. The temperature of the glass slide was maintained at 38-40°C. At magnification of 10x was used for microscope examination. Light and dark waves of spermatozoa swirling over the field of focus were observed. The number of eddies passing through the focused field in 15 seconds were recorded and the neat semen was evaluated as per the following table.

Table 2 Grading of neat semen

Type of Wave motion	Eddies/15 sec	Approx. initial motility (%)	Grade
Less motility	0	5-10	Dead, 000
Slight motility, no wave motion	0	10-20	Very poor,00
Not clear waves/eddies. Some throbbing movements.	<1	20-40	Poor,0
Not clear waves/eddies. Oscillating and circulating spermatozoa in the field.	1-2	50-60	Fair,+
Slow moving waves/eddies appeared scattered in field	2-3	60-70	Better,++
Not so rapid waves/eddies. Swirls moving towards periphery.	3-4	70-80	Good,+++
Extremely rapid waves/eddies. Origin and disappearance of waves is unclear.			

Sperm concentration

The concentration of sperms was measured using the direct cell count method using hemocytometer[9]. Semen was diluted by normal saline at a dilution rate of 1:200. The haemocytometer was charged with this diluted semen at magnification of 40X. The concentration of sperm per volume was found using the formula:

Concentration of semen per mm^3 of diluted semen = 50000 × Number of spermatozoa counted Dilution rate.

Estimation of different oxidative stress-related biochemical parameters in testes was carried out. The lipid peroxidation in testis was determined in terms of malondialdehyde (MDA) production by the method [10]. GSH was estimated by the 5, 5' dithiobis (2-nitrobenzoic acid) (DTNB) method [11]. The activity of glutathione peroxidase (GPx) [12]. A double beam UV-VIS spectrophotometer was used for recording the absorbance of the test samples.

Gross and histopathological examination

Three birds from each group were sacrificed using standard ethical procedures 16 weeks post treatment (WPT). All the birds were subjected to detail post mortem examination and the gross lesions, if any, were duly recorded. Representative sample testis were collected in 10% neutral buffered formalin for histopathological examination and processed as per standard protocol[13].

Electron microscopic studies

The sample for ultra-structural study of testis of group II, III, IV, V, VI and VII were be collected, processed and analyzed in the Electron Microscopy Lab, Department of Anatomy, All India Institute of Medical Sciences, New Delhi by standard process.

Statistical analysis

Statistical analysis was done using two way analysis of variance (ANOVA) to determine the significance difference ($P < 0.01$) in efficacy of HSRP treatment between two means based on individual observations of different parameters[14].

Results and Discussion

The mass motility of the sperms and sperm count (10^9/ml) reduced significantly ($P<0.0$) in indoxacarb treated group II in comparison to control group I(Table 3). In groups VI and VII showed significant ($P<0.05$) restoration in sperm motility towards normal similar to group IV treated with silymarin. This might be due to the tonic and vasodilator effect of *Hedychium spicatum*. The indoxacarb was reported to reduce the fertility and fecundity indices in rat[15]. Reduction the motility also might have occurred to other exhaustion of metabolic energy or its production due to interference in oxidative glycolysis in sperm. Indoxacarb induces the oxidative stress created cellular toxicity and increased vulnerability of spermatozoa to oxidative stress due to low activity of antioxidant enzymes and elevated concentration of poly unsaturated fatty acid (PUFA) in spermatozoa or seminal plasma. Generation of reactive

Table 3: Effect on mass motility following administration of HSRP in feed for 16 weeks in indoxacarb treated WLH cockerels (Mean±SE, n=7).

Groups	Treatments	Mass motility	% Dead	Sperm count (10^9/ml)
I	Control	3.62 ± 0.062^{b}	24.85 ± 0.83^{e}	2.28 ± 0.063^{b}
II	Indoxacarb(250ppm)	2.32 ± 0.038^{e}	41.42 ± 0.44^{a}	1.37 ± 0.026^{f}
III	Silymarin(250ppm)	3.80 ± 0.28^{a}	23.00 ± 0.53^{f}	2.48 ± 0.024^{a}
IV	Indoxacarb(250ppm)+ Silymarin(250ppm)	3.42 ± 0.069^{c}	28.28 ± 0.38^{d}	1.97 ± 0.056^{c}
V	*H. spicatum*(4000ppm)	3.70 ± 0.034^{ab}	24.57 ± 0.44^{e}	2.35 ± 0.048^{b}
VI	Indoxacarb(250ppm)+ *H. spicatum*(2000ppm)	3.22 ± 0.038^{d}	34.42 ± 0.52^{b}	1.72 ± 0.038^{e}
VII	Indoxacarb(250ppm)+ *H. spicatum*(4000ppm)	3.31 ± 0.031^{cd}	31.71 ± 0.56^{c}	1.84 ± 0.018^{d}

HSRP: *Hedychium spicatum* root powder; Mean values in column bearing different alphabetic superscript a, b, c, d, e and f differ significantly ($p<0.05$) when compared vertically.

oxygen species (ROS) and peroxidation of sperm membrane may produce detrimental effects on sperm motility, midpiece abnormalities and sperm-oocyte fusion[16, 4].

In groups VI and VII treated with the indoxacarb+HSRP showed significant ($P<0.05$) elevation in sperm count in comparison to indoxacarb group II. This amelioration might be due to the antioxidant property of the extracts which have beneficial effect on sperm motility by inhibiting the lipid peroxidation of the sperm membrane. Indoxacarb was found to reduce the fertility and fecundity indices in rats [15].

There was significant ($P<0.05$) elevation in MDA concentration and GPx activity in testis in indoxacarb intoxicated group II comparison to control group I at 16 weeks intervals testes (Table 4) [3]. In groups VI and VII treated with indoxacarb+HSRP at both low and high doses decreased significantly ($P<0.05$) in level of MDA at 16 weeks interval in comparison to group II and which was at par with value in control and silymarin groups I and IV, respectively. A significant ($P<0.05$) reduction of GSH activity in indoxacarb intoxication and simultaneous feeding of HSRP in group VI and VII improve the GSH activity in dose dependent manner indicates have hepatoprotective activity of HSRP[17].

The significant reduction of MDA level and GPx activity by treatment of HSRP, showed amelioration in LPO and GPx activity which might be due to its potent antioxidant activity due to presence of flavonoids and other phyto-constituents in rhizomes of *Hedychium spicatum*[18, 17].The flavonoids and phenolic compounds, exhibited high antioxidant and free radical scavenging activities in rats [5].

The elevation in the concentration of MDA, an indicator of lipid peroxidation in the testis clearly indicated that the indoxacarb induced oxidative stress during 16 weeks exposure in cockerels. Enhanced peroxidation of lipids in intra and extracellular membranes results in the damage to the cells, tissues and organs. Enhanced lipid peroxidation leads to peroxidation of polyunsaturated fatty acids and subsequent damage to lipid of the membrane [19].

In the present investigation, the cause for increased MDA level in testes under the influence of indoxacarb treatment might have been due to the conjugation of indoxacarb or its metabolites to the polyunsaturated fatty acids or by production of ROS react with polyunsaturated fatty acids or accumulation of lipophilic components of pesticides conjugated with-the fatty acids [3].

No microscopic changes were seen in the testes of birds of group I (control) and III (Silymarin@ 250 ppm). In group II birds, there was severe reduction in the germinal epithelium in the basement membrane. Severe decline in the number of spermatid in almost all the seminiferous tubules and decrease in size of the majority of the seminiferous tubules (Plate 1) were also noticed[20, 21]. These lesions were of very mild to moderate intensity in testes of birds of group IV (Silymarin+Indoxacarb), V (HSRP @ 4000ppm), VI (HSRP @ 2000 ppm + Indoxacarb @ 250 ppm) (Plate 2) and VII (HSRP @ 4000 ppm + Indoxacarb @ 250 ppm).

The lesions caused in testes in this study caused by indoxacarb may be attributed to oxidative damage caused by increased production of highly reactive oxygen and nitrogen species. The ameliorative effect shown by *Hedychium*

Table 4: Effect on antioxidant parameters in tissues following administration of HSRP in feed for 16 weeks in indoxacarb treated WLH cockerels (Mean±SE, n=7).

Groups	Treatment	LPO(nM MDA/mg tissue)	GSH(mM GSH / mg tissue	GPx (U/gm tissue)
I	Standard ration *ad lib*	143.28±1.11^{b}	0.81±0.044^{a}	5.68±0.65^{b}
II	Indoxacarb (250 ppm)	156.33±2.93^{a}	0.36±0.026^{c}	7.08±0.15^{a}
III	Silymarin (250 ppm)	143.15±3.04^{b}	0.80±0.062^{a}	5.72±0.53^{b}
IV	Indoxacarb (250 ppm) + Silymarin (250 ppm)	145.50±2.32^{b}	0.63±0.084^{ab}	5.97±0.21^{ab}
V	*Hedychium spicatum* rhizome powder-(HSRP) @ 4000ppm)	143.25±2.55^{b}	0.80±0.076^{a}	5.67±0.46^{b}
VI	HSRP@ 2000ppm+ indoxacarb@250ppm	147.41±2.05^{b}	0.54±0.051^{b}	6.39±0.22^{ab}
VII	HSRP@ 4000ppm+ indoxacarb@250ppm	146.24±2.69^{b}	0.60±0.073^{b}	5.99±0.16^{ab}

HSRP *Hedychium spicatum* root powder; Mean values in column bearing different alphabetic superscript a and b differ significantly ($p<0.05$) when compared vertically

spicatum(HSRP) might be due to the presence of polyphenols which attribute to the antioxidants[22], immunoprotective[23,24] and hepatoprotective[17] properties of the plant.

Ultra structure of testes showing decapitated spermatozoa and lumen of seminiferous tubule filled with numerous cross sections of sperm tails. The testes showing reduced number of spermatogonial cells in indoxacarb intoxicated groups II (Plates 3).

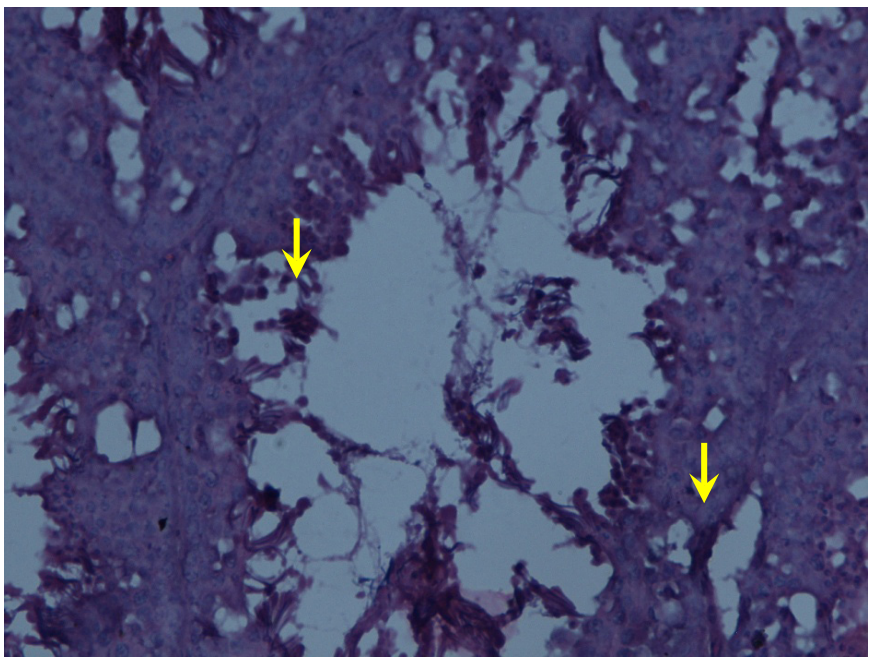


Plate 1: Testes showing necrosis and depletion of majority of spermatids from majority of the seminiferous tubules (x 400, HE) Group II (Indoxacarb @ 250 ppm).

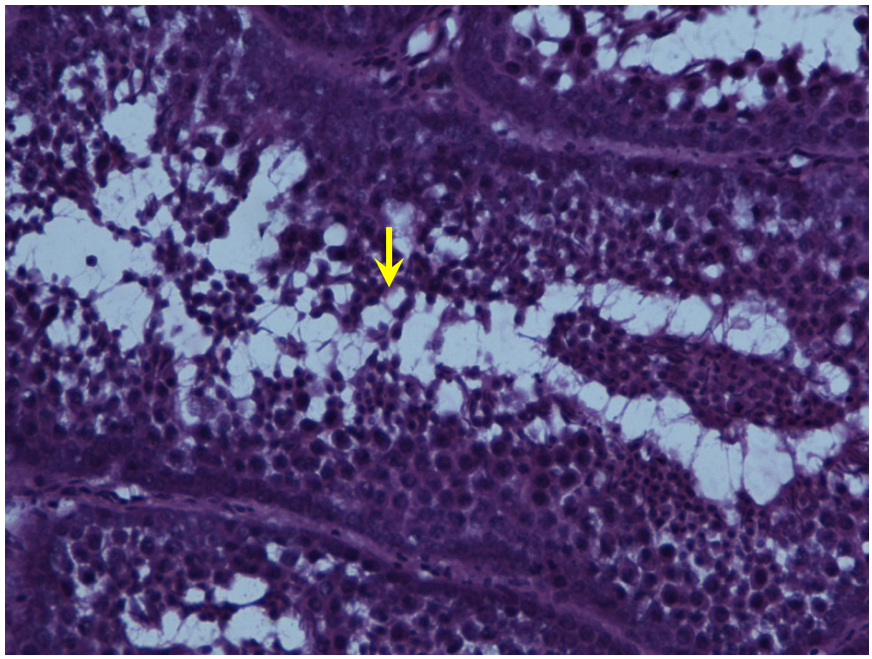


Plate 2: Testes showing moderate depletion of spermatids from the lumen of the seminiferous tubules (x 400, HE) Group VI (Indoxacarb @ 250 ppm + HSRP @ 2000 ppm).

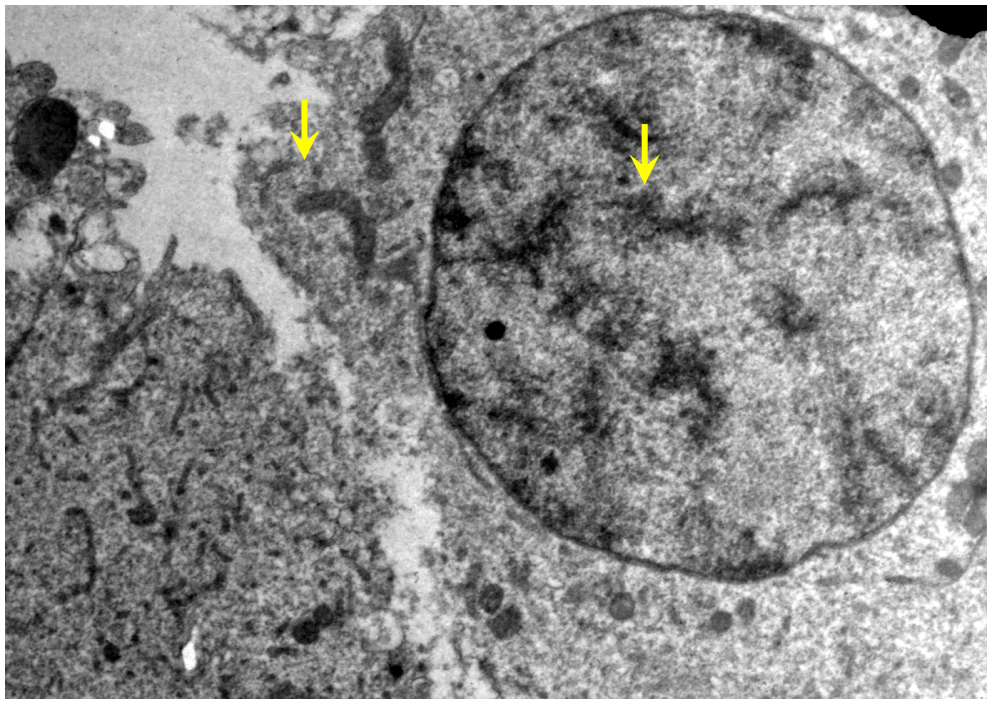


Plate 3: Ultra structure of testes showing decapitated spermatozoa and lumen of seminiferous tubule filled with numerous cross sections of sperm tails (x 8000) Group II (Indoxacarb @ 250 ppm).

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Conflict of Interest

None Declared

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