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REPRODUCTIVE TOXICITY OF AN ORGANOPHOSPHORUS PESTICIDE ON MALE FROGS (*BUFO MELANOSTICTUS*)

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Abstract: Global decline of amphibians have been of international concern and agrochemical pollutants, including those that are potential disruptors of sexual development, are suspected to be one of the most important contributory factor. Due to the biphasic life cycle and susceptibility to environmental contaminants, amphibians are good bio indicators and their decline has sparked concern over environmental degradation. The aim of this study is to evaluate the effects of sub lethal concentrations of Hinosan on the testes histoarchitecture and Gonadosomatic index of *Bufo melanostictus* Schneider. Adults were exposed to two sub lethal doses (1/10 and 2/10 of Lc50) of Hinosan for a period of 30 days .A control group without exposure to Hinosan was also maintained. *Bufo melanostictus* is a continuous breeder and control group testes showed oval intact seminiferous tubules with all the different stages .Up to ten days, testes of those exposed to lower concentration showed more or less normal structure, whereas changes were evident in the testes of those exposed to higher concentrations from the fifth day itself. But after ten days of exposure, pronounced changes were observed in the histology of testes of all the treatments. Degenerative changes in the seminiferous epithelium, histological changes in reproductive organs, testicular atrophy, tubule shrinking, and necrosis of spermatogenetic cells, exfoliation and agglutination of sperm bundles, clumping of chromatin material etc. were noticed followed by a reduction in the gonadosomatic index. The structure of the testes of pesticide exposed *Bufo* was completely disorganised and also an imbalance in the proliferative activity exhibited, further confirmed the lethality of pesticides on the non target organisms like frogs.

Key words: Amphibians, Decline, Agrochemical pollutants, Sub lethal concentration, Sexual development, Histoarchitecture, Degenerative changes, Testicular atrophy

INTRODUCTION

Agrochemicals are being lavishly used in India in agricultural operations to curtail pest menace and increase production. Several of them are unselective and toxic and their indiscriminate use disrupts the ecosystem health which results in an imbalance between organism and their environment (Chukwu, *et al*., 2003; Saunders and Harper, 1994; Casas *et al*., 2010). High solubility, mobility and intensive use of these pesticides may contaminate the ecosystem, and their application usually coincides with the breeding and development of nontarget organisms like amphibians (Lenkowski *et al*., 2008; Matsuhita *et al*., 2006; Rohr *et al*., 2006; Gilbert and Bolker, 2001; Dutta and Sahu, 2013). Amphibian population decline at an alarming rate has been documented from several parts of the world and anthropogenic factors like agrochemical contamination, habitat loss, overexploitation, introduced species etc. are suspected to be the potential causes for this decline (Wake, 1991; Gallant *et al*., 2007; King *et al*., 2010; IUCN 2006; Stuart *et al*., 2004; Beebee and Griffith, 2005; Kiesecker *et al*., 2004, Song, *et al*., 2002). Because of their biphasic life cycle and semi permeable skin, they fall easy victims to these exogenous contami-nants and hence are good bio indicators of environmental degradation (Hall and Henry, 1992; Cooke, 1981; Boyer and Grue, 1995; Carey and Bryant, 1995; Fenoglio *et al*., 2009).

Organophosphorus pesticides are a preferred alternative for organochlorines because of their law persistence and rapid degradation .However they have greater acute toxicity even at law levels of exposure(Blasiak *et al*., 1999) and have been in use in large quantities for a long time to control both vertebrates and invertebrates (Debleecker *et al*., 1993; Sparling and fellers, 2007; Hill, 2003). Recent studies sparked concern over the fact that these chemicals in combination with that of their degradation cause serious toxic

effects on the meiotic maturation and normal reproductive health of frogs (Sauco *et al*., 2010) Terratogenecity of these xenobiots on metamosphosis and early developmental stages can affect the later developmental stages including gonad development in frog since organogenesis takes place in early stages (Chenkowski *et al*., 2008). Eventhough the effect of organophosphrous pesticides were considered to be considerably less in vertibrates, there are evidences for their serious effect on germ cell development (Bustos-obregon and Gonzalez-Hormazobal, 2003; Cases *et al*., 2010). Reduced sperm motility reported in rats exposed to an organophosphate insecticide Diazinon (Abd el-Aziz *et al*., 1994). Alteration in testicular histoarchitecture in *Caiman Lairostris* exposed to Agrochemical pesticides has been reported by Rey *et al*. (2009). Extensive literature is available on the systematic toxicological studies focused on the histochemial and histopathological effects of these exogenous chemicals on other vertebrates. Such studies on amphibians are very scarce (Freeman and Rayburn, 2004; Hecnar, 1995; Power *et al*., 1989; Cakici, 2013).

Paddy is the main agricultural crop in the Southern part of Kerala. Kuttanad, once the "Rice bowl" of Kerala is now "poison bowl", due to the indiscriminate use of pesticides, mainly organophosphorus insecticides and fungicides. Hinosan (Ediphenphospho-ethyl-SS-diphenyl-phopherodithioate), an organophosphorus fungicide, marketed by Bayer AG in 1968 is widely and commonly used to control *Pyricularia oryzae* on rice (Scheinpflug and Jung, 1968). Adverse effects of Hinosan on the hatching rate and larval survival and growth rate on tadpoles of *Bufo melanostictus* has been stated by Mathew and Andrews (2000, 2003). Hence in the present study, an attempt has been made to evaluate the toxic effects on gonadosomatic index and histoarchitecture of the gonad of male *Bufo melanostictus* exposed to sub lethal concentrations of Hinosan, an organophosphorus fungicide.

MATERIALS AND METHODS

Hinosan was procured as a 50%w/w emulsifiable concentrate from Bayers (India) Ltd. Bombay, India. A stock solution of Hinosan was prepared by adding 2ml. of the chemical to 1 litre of pure ultra-filtered sterilised water. The stock solution was prepared weekly or as needed. Doses were prepared by calculated dilution of the stock solution with water.

Adult male toads were collected from regions where there were no agricultural activities or industries in the nearby locality. Male toads weighing between 25-35 gms were used for the present study. They were acclimated to laboratory conditions for about a fortnight and were fed with fresh liver every second day. No mortality was observed during the period of acclimatisation. Animals were intraperitoneally injected with different concentrations/gm. bodyweight. The mortality rate of toads for 24 hours was recorded LD50/gm body weight for Hinosan was determined by Probit analysis (Finney, 1971).

For the toxicological studies three groups of (20 each) toads were selected. Group-1 served as control. The experimental groups, Groups 2 and 3, received a low dose 1/100f LD_{50} (0.054 ppm/ gm body weight) and median dose 2/10 of LD_{50} (0.108 ppm/gm body weight) respectively. Intraperitoneal injections were given weekly to maintain the concentration of the pesticide steadily. The control group was injected with one ml of distilled water. After exposure, four toads each from each group anesthetised and sacrificed at an interval of five days. In-vivo perfusion of testis with Bouins fixative was used for fixation of testis. Testes tissue was processed and embedded in paraffin wax. Five-micrometer thick sections were made and stained with Haematoxylene and Eosine. The experiment was maintained up to thirty days.

$$GSI = \frac{\text{Weight of the gonad}}{\text{Weight of the body}} \times 100$$

Gonadosomatic index was calculated using the formula for each individual toad.

Cell nests were counted from twenty randomly selected sections of the testis of eachanimal. Spermatogenetic stages were identified as described by Van Oordt (1956) and a slightly modified version of classification was followed as

described by Saidapur (1983).The different stages are as follows: stage0-Primary spermatogonia,stage1-secondary spermatogonia, stage, 2: primary spermatocytes, stage 3-secondary spermaocytes,stage4: spermatids,stage5- sperm bundles attached to sertoli cells.

RESULTS

During the pilot study conducted to determine the rate of mortality, the toads exposed to Hinson showed restlessness, and by the advancement of time, responses to touch and sound were found decreased. Coagulation of mucus and peeling of skin was frequently found in toads exposed to higher concentration. Blotting of belly and imbalance in jumping noted. The advanced stages of intoxication were evidenced by their alarming calls and blind movements. The LD_{50} 24 hour for adult toads for Hinson was found to be 0.54ppm/gm body weight and LD_{100}24 hour was 0.8 ppm/gm body weight. Fiducial limits were 0.49ppm to 1.24ppm and regression equation was $Y=2.48+3.5x^2$ value was 26.25 and was significant.

The transverse sections of control testis showed normal structure. Each tubule is at the height of spermatogenetic activity and contained primary and secondary spermatogonia and spermatocytes, spermatids and spermatozoa, sertoli cells and interstitial cells of Leydig. Each seminiferous tubule is lined by a thin basement membrane and the interstitial cells of Leydig and connective tissues present in between the tubules. The spermatozoa could be seen in bundles in the lumen of the seminiferous tubules (Plate 1, A and E).

Histopathological changes in the testes of treated toads

Exposure to 0.054 ppm/gm body weight of Hinson

Up to fifteen days the lumen and the tubules were roughly the same even though slight enlargement was evident. Histopathological changes became evident after fifteen days of exposure. When compared to control they were less organized .Clumping of chromatin material of the primary spermatocytes and pyknosis were also noted. On the 20th day secondary spermatocytes showed degeneration and Leydig cells showed size variations. At 25 days congestion in the interstitial cells was recorded. Tubules were highly dilated and cellular debris noted in the lamina and seminiferous tubules were broken in many places. Leydig cells showedegenerative changes. Changes were all highly significant. Spermatids and spermatocytes were damaged. After thirty days of exposure, there was disruption in the cystic nature of the spermatogenetic stages. Tubule boundaries were lost and necrosis and reduction in the number of germ cells noticed. Connective tissue was less pronounced (Plate 1. B, C and D).

Exposure to 0.108ppm/gm body weight

After five days of exposure, vacuolation and scattering of sperm bundles seen in 20% of tubules. Testis showed mild degenerative changes after ten days of exposure. The sperm bundles were disorganized and sperms were scattered. After fifteen days, vacuolation within the epithelial layer intensified. Nuclei of spermatogonial cells were enlarged. Exfoliation and aggregation of cells and reduction in the number of mature sperms were also noticed. Degenerative changes were more pronounced. Seminiferous tissue walls were missing in many areas. After twenty days of exposure, changes were all highly significant. Degenerative changes were more pronounced in most of the tubules. Disorganisation in more than 75% of the tubule / toad has been noticed after 25 days of exposure. Swelling and sloughing of germ cells and size variation and clumping of Leydig cells were observed. Pyknosis, karyorhexis and lysis were also noted in the Leydig cells .Thirty days of exposure produced haemorrhagic necrosis in the tubules. Several degenerative changes and absence of distinct cell organisation were recorded. Leydig cells were harder to find. All the degenerated tubules showed necrosed spermatogenetic cells and the lumen was devoid of active sperms. (Plate 1 F, G and H). Marked reduction from the control value has been noticed in the GSI of toads at 5,10,15,25 and 30 days exposure to both concentrations of Hinosan(Table 1).The results were statistically analysed and found to be highly significant in all the treatments ($P<0.01$).

Table 1. Effect of Hinosan on the GSI of *Bufo melanostictus*

Conc	5thday	10thday	15thday	20thday	25thday	30thday
Control	2.17+0.060	0.32+0.067	0.15+0.012	0.15+0.021	0.21+0.037	0.31+_0.088
0.054ppm	0.40+0.034	0.29+0.025	0.10+0.004	0.11+0.018	0.13+0.037	0.20+0.021
0.108ppm	0.38+0.048	0.13+0.008	0.06+0.011	0.07+0.005	0.03+0.004	0.12+0.032
VR	440.32**	5.94**	6.7**	5.9**	8.66**	7.2**
CD	0.15	0.03	0.06	0.07	0.15	0.18

** Significant for P < 0.01

Table 2. Effect of Hinosan on spermatogenetic stages of *Bufo melanostictus*

Stage	Status	5days	10days	15Days	20Days	25days	30days
Stage0	Control	0.25±0.17	0.65±0.20	1.7±0.27	0.7±0.15	1.4±0.06	0.25±0.17
	Treated	0.15±0.13	0.10±0.3	0.13±0.03	0.08±1.15	0.03±0.03	0.01±0.03
Stage 1	Control	0.5±0.11	1.11±1.1	0.68±0.08	2.7±1.32	2.5±0.07	1.9±0.22
	Treated	1.65±0.44	0.65±0.07	0.61±0.30	0.65±0.05	0.78±0.01	0.14±0.17
Stage 2	Control	1.05±0.04	1.15±0.20	2.0±0.07	1.5±0.34	1.65±0.19	1.9±0.81
	Treated	2.23±0.8	2.27±0.06	1.85±0.32	0.9±0.25	0.25±0.38	0.21±0.51
Stage 3	Control	2.85±0.18	3.35±0.36	2.2±0.19	1.9±0.19	2.5±0.12	2.5±1.1
	Treated	0.93±0.26	0.8±0.08	1.15±0.27	0.48±0.33	0.38±1.8	0.37±1.3
Stage 4	Control	2.65=0.24	3.3±0.18	2.85±0.04	1.7±0.16	2.7±0.06	3.5±0.38
	Treated	2.63±0.18	1.65±0.16	1.15±1.16	0.88±0.06	0.80±1.3	0.25±1.25
Stage 5	Control	16.95±1.93	15.95±2.6	13.85±1.2	14.75±0.4	10.3±1.4	11.0±1.2
	Treated	6.38±0.07	6.32±0.37	3.85±1.4	3.21±0.78	0.78±1.35	0.08±1.26
	VR	26.99**	43.11**	24.89**	17.06**	7.43**	6.42**
	CD	0.42	0.59	0.33	0.18	0.38	0.57

** Significant for P < 0.01

Effect on spermatogenetic stages

The frequency distribution of spermatogenetic stages in the Hinosan treated toads are shown in table 2.Up to five days there were not much significant decrease in the different cell stages but slight increase has been noted in secondary spermatogonia and primary spermatocytes. After ten days of exposure significant decrease has been noted in all the cell stages except secondary spermatocytes which showed an increase when compared to those of the control. Primary spermatogonia decreased in comparison to the control and the changes were highly significant. Spermatozoa showed a highly significant decrease in all the treated toads. The proliferation of primary spermatogonial cells affected in all the treated frogs but, the proliferative activity of secondary spermatogonial cells was very significant, as their distribution was very much reduced on the 30th day of exposure.

DISCUSSION

The present study clearly shows the various pathological effects on the testis of *Bufo melanostictus* exposed to sub lethal doses of hinosan. Histopathological effects were dose and duration dependent and these could negatively affect the spermatogenic process in this anuran species.Herpetologists and environmentalists have voiced concern over the declining amphibian populations including endangered species in many parts of the world (Blaustein and Wake, 1994; Alford and Richards, 1999; Houlahan *et al.*, 2000; Freeman *et al.*, 2004; Bell *et al.*, 2004) and habitat loss and environmental degradation are considered to be the main reasons for the decline (Delis *et al.*, 1996; Semilitsch. 1998; Semilitsh and Bodie 1998). But agriculture is one single activity ,considered to be the main reason for the decline in endangered and sentinel species of amphibians(Saunders and Harper 1994; Casa *et al.*, 2010; Houlahan *et al.*, 2000; Kolozsvary and Swihart, 1999; Blautein and Keisecker, 2002; Rey *et al.*, 2009.

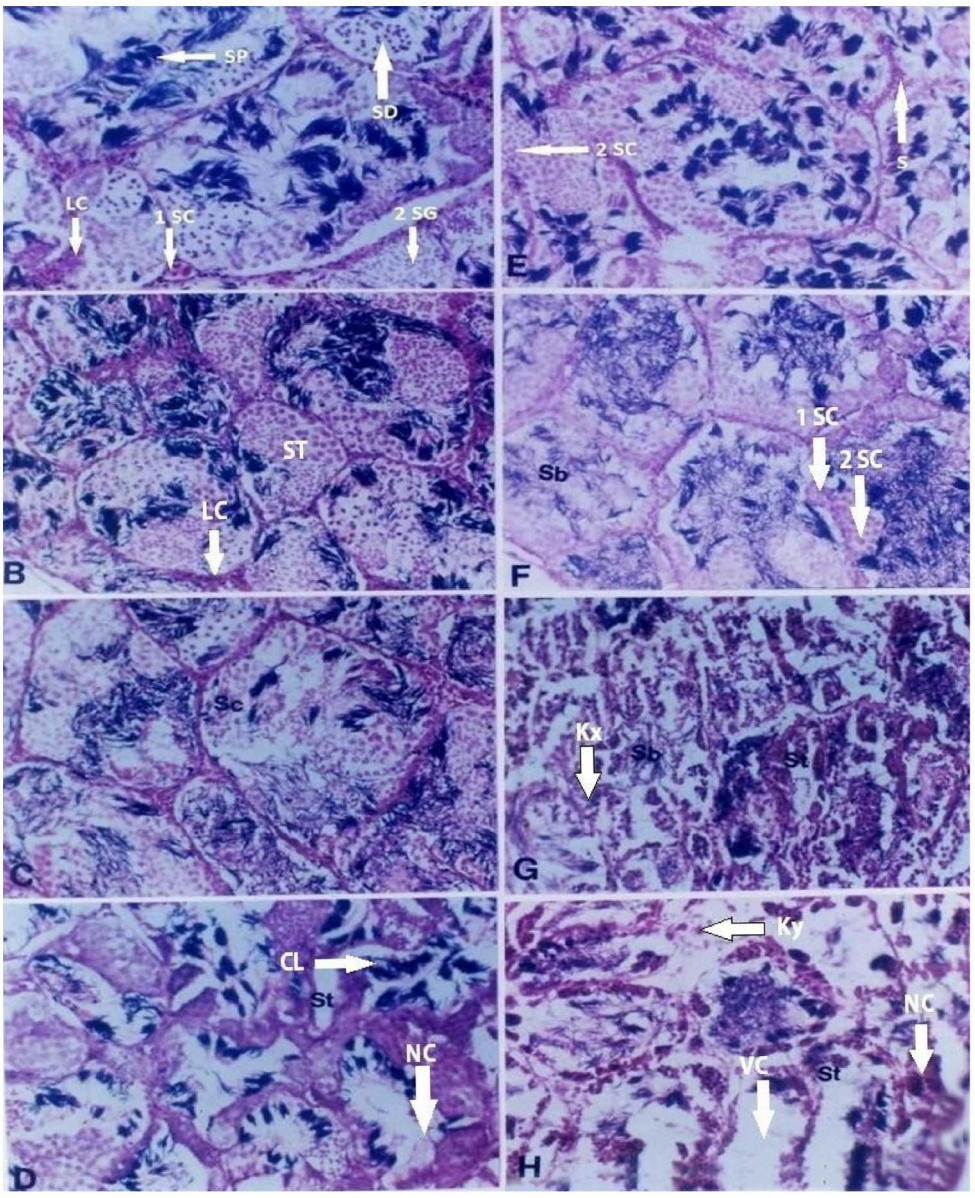
Both heavy metals and pesticides are known to affect the testis in vertebrates and cause significant reduction in the Gonadosomatic index (Seghal and Pandey 1984). Exposure of fishes to heavy metals like cadmium (Wany and Latey, 1982; Seghal and pandey, 1984) and lead (Katty and Sathyanesan, 1985) produced severe testicular damage and reduction in the GSI values. Testicular weight loss was recorded in fishes exposed to Dimecron (Lakhani and pandey, 1985), BHC (Pandey and Shukla, 1980), and fenitrothion and carbofuran (Saxena and Mani, 1985). Among the amphibians, testicular weight loss and necrosis following intratesticular injection of cadmium were recorded in *Rana tigerina* (Setty and Kar, 1964). Decrease in GSI value has been reported in *Rana cyanophlyctis* exposed to Emissan, a fungicide (Kanamadi and Saidapur, 1992) and *R. hexadactyla* exposed to malathion (George and Andrews, 1994). Chairat *et al.* (2003) reported decrease in the Gonadosomatic index of *Hoplobatrachus rugulosus* exposed to methyl parathion and the decrease noted was significantly higher when compared to those of control. The present study reveals significantly higher reduction in GSI of Adult male *B. melanostictus* exposed to sub lethal concentrations of Hinosan. Gonadosomatic index is directly proportional to sexual ability and normal spermatogenetic cycle, and reduction in GSI may be indicative of the disrupted sexual activity (Chairat *et al.*, 2003)

Definite testicular injury and spermatogenetic arrest following treatment with pesticides have been reported for representative vertebrates. Degenerated tubules with necrosed spermatogenetic cells and the absence of active sperms in tubules have been reported in the testis of guinea pigs exposed to Benzene Hexachloride (Dikshit *et al.*, 1978).Various pathological symptoms including vacoulation, degeneration of sperms ,reduction in the size and number of spermatogonia, were observed in fishes exposed to different pesticides (Jyothi and Narayan, 1996; Zutshi and Murthi, 2001). BHC produced hypertrophy in both primary and secondary spermatocytes in *Tilapia mossambica* (Lakhani and Pandey, 1985). The pesticides like endosulfan can cause disorganisation and destructive changes in the spermatogenetic stages in Blue gill fish, *Lepomis macrochirus* (Dutta *et al.*, 2006). Singh and Sahai (1987) reported testicular degeneration and disappearance of sperms in 15 days in *Heteropneustus fossilis* exposed to endosulfan. Reduction in the size of breeding gland, decreased level of testosterone, chemical castration etc have been observed in *Xenopus laevis* males exposed to atrazine (Hayesa *et al.*, 2006; 2010). Alterations in the male reproductive activity has been stated by Dutta and Sahu (2013) in rats exposed to an OP pesticide chlorophyriphos. Campagna *et al.* (2002) reported that exposures to different organochlorines can reduce fertility by affecting the viability and motility of sperms in pigs.

The organophosphorus pesticides has been listed as chemicals highly toxic to both invertebrates and vertebrates including humans (DeBleecker *et al.*, 1993). A large no of OP pesticide formulations are now available (Sparling and Fellers, 2007; Hill, 2003) and is the largest and most widely used group of pesticides with acute toxicity potential for the reproductive system. (Astroff *et al.*, 1998; Nguola *et al.*, 2007). Maitra and sarkar (1991) stated gametogenetic disorders in *Psittacula krameri* and *Longura malabaricus* exposed to quinalphos. Disruption of the cystic nature of the spermatogenetic stages and scattering of sperms in the lumen have been reported in *R. hexadactyla* exposed to malathion (George and Andrews, 1994)

Hydrophic swelling, pyknosis, karyorhexis and lysis, disruption of nucleus and clumping noticed in the Leydig cells in the present study. By the advancement of exposure period, the degenerative changes were well pronounced and their number highly reduced. Any kind of damage to Leydig cells will affect the testosterone level and may lead to disruption in the spermatogenetic activity (Archana *et al.*, 2007; Rattner and Michael, 1985; Smallridge *et al.*, 1991). Histopathological changes noticed might be due the degenerated leydig cells. Disorganisation and alteration of cell may affect the synthesis and secretion of testosterone. Altered spermatogenesis, distortions in seminiferous tubules, oedema of interstitial cells, loss of sperms etc were observed in *Hoplo-*

Photomicrographs of sections of testis of B. melanostictus exposed to Hinosan (X 250)
A & E - Control, B, C, & D - Exposed to 0.054ppm, F, G, & H - Exposed to 0.108ppm



B,F – 10th day; C,G – 20th day D,H – 30th day

1 Sc – Primary spermatocytes, 2 Sc – Secondary spermatocytes, 2 Sg – Secondary spematogonia, SD – Spermatids, SP – Sperm bundle, ST- Seminiferous tubules, S – Sertoli cells, LC – Leydig cells, VC – Vacoulation, CL – Clumping, NC – Necrosis, Kx – Karyorhexis, Ky – Karyolysis.

batrachus rugulosus exposed to methyl parathion(Chairat *et al*., 2003), testis of albino rats exposed to carbaryl (Archana *et al*., 2007), primiphosmethyl (Nguola *et al*., 2007) and malathion (Bustos-Obregon, E. and Gonzalez-Hormazabal, 2003). Exposure of bispenol A has affected the steroidogenesis in the Leydig cells of mouse testis (Song *et al*., 2002). Disturbed spermatogenesis, damage to connective tissue, oedema of the interstitial walls and loss of sperms of varying degrees have been observed in *Bufo melanostictus* males exposed to Hinosan.

Similar effects have been reported in male rats exposed to carbaryl (Archana *et al*., 2007) and Primiphos-methyl (Nguola *et al*., 2007). Findings of the present study is consistent with those found in other vertebrate classes and confirms that the effects of OP pesticides are not species specific but can occur in any group of vertebrates.

Development of gonad and its functioning is controlled by hormones and the influence of testosterone is well known. These toxicological changes may be due to the degeneration of Leydig cells, which in turn inhibit the testosterone production (Ratter and Michael, 1985; Smallridge *et al*., 1991). Histopathological changes in the testis of organophosphate pesticide treated animals may be attributed to endocrinological (Civen *et al*., 1977; Muller *et al*., 1977; Colborn *et al*., 1993; 1996; Anderson *et al*., 2000; Mathur *et al*., 2010) and/or pharmacological (Chakraborthy and Nelson, 1976; Goodman and Harbinson, 1981) actions of the administered organophospherous pesticide. The inhibition of acetyl cholinesterase activity has been seen as indicative of OP poisoning (Martin *et al*., 1981; Pope, 1999; Cabello *et al*., 2001; Colombo *et al*., 2005; Sparling and Fellers, 2007). Any inhibition in acetyl cholinesterase activity may block nerve impulse and can lead to suppression of hormonal release (Gwynne, 2000). Fungicide Hinson contains ethyl group attached to the phosphorus. Inside the animal, the phosphorothionates are converted to phosphates, which bind with acetyl cholinesterase enzyme that breaks down the neurotransmitter acetylcholine (Fest and Schmidt, 1983). Besides Organophosphates can cause functional alterations in the internal organs like kidney and liver and toxic substances drained from there to circulatory fluid can disrupt spermatogenesis and germ cells(Lox, 1985; Kiran *et al*., 1985; Archana *et al*., 2007). Findings of these studies shows that gametogenic functions in general are not free from cholinergic systems (Goodman *et al*., 1984). So there are reasons to believe that the degree of histopathological changes in the testis of *Bufo melanostictus* exposed to Hinson may be related to the degree of Ache activity in the central nervous system (O'Brien, 1967).The results thus obtained clearly indicative of the disruptions caused to the spermatogenesis in general which can lead to impairment of reproduction and loss of fertility in *Bufo melanostictus* males exposed to Hinson.

CONCLUSIONS

There is sufficient evidence to believe that amphibian population decline is a fact and agricultural operations including indiscriminate use of pesticides can be attributed to this alarming situation. Amphibians will be exposed to these contaminants by absorption through skin, inhaling contaminated air, feeding or by surface run off of these chemicals to the ponds where they breed. The impacts of unsustainable agricultural practices in combination with effects of climate change have to be identified and sorted out at the initial stages before they become uncontrollable. The observations of the present study states that Hinosan, an organophosphorus fungicide could disrupt the spermatogenetic process which may affect the reproductive health.To redress the effects further investigation is required to ascertain the metabolism and accumulation of these contaminants in tissues which would be species specific.

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