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EFFECT OF THREE COMMONLY USED INSECTICIDES ON HISTOMORPHOLOGY AND HISTOCHEMISTRY OF TESTIS OF EARTHWORM EUDICHOGASTER KINNEARI (ANNELIDA: OLIGOCHEATA)

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ABSTRACT :

Adult Eudichogaster kinneari were exposed to 0.6 ppm concentration of Dimethoate, 0.5 ppm concentration of Azodrin and 0.003 ppm concentration of Thiodan for twenty days to evaluate profound changes in the histomorphology and histochemistry of testis. Spermatogenesis was severely affected with exposure of above insecticides causing reduced testicular activity by destruction of cellular architecture observed by vacuolization of cells in different stages of spermatic follicles and in cytophore, necrosis, lesions, congregation of spermatogenic material, and uneven arrangement of spermatozoa around the cytophore and ultimate atrophy of spermatic follicles. Decreased intensity with histochemical reactions and reduced size of spermatic follicles ($p<0.001$) were observed. The present study indicates that among the three insecticides tested, thiodon is most toxic to earthworm E.kinneari, than azodrin and dimethoate respectively. The intensity of deterioration were noticed more toxic in thiodan > Azodrin > Dimethoate respectively.

KEY WORDS: Eudichogaster kinneari, Insecticide, testis, histomorphology, histochemistry.

INTRODUCTION :

Earthworms are indicators of soil quality because they respond to and contribute to healthy soil. They benefit soil quality by shredding residues stimulating microbial decomposition improving soil fertility and improving physical properties of soil such as soil aggregation and infiltration. Food availability is the major limiting factor for earthworm numbers. Generally fertilizers increase earthworm numbers by increasing crop residues, especially when pH is neutral. However, some

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insecticides, nematicides and fungicides are very toxic to earthworms (Edwards and Bohlen 1996).

Earthworms are considered as important bioindicators of chemical toxicity in the soil ecosystem and play a key role in the biomagnifications processes on several soil pollutants (Cikutovic et al. 1993, 2010;celine et al. 2014) Soil pollution enormously increased due to intensive use of fertilizers, pesticides and insecticides for betterment of agricultural yield. They ultimately persist in soil and decrease soil fertility, causes disturbance in balance between flora and fauna residing in the soil. In this way agrochemicals not only affect the insects but equally damage the soil fauna. Inspite of this, there is lack of information on the effect of three commonly used insecticides dimethoate, azodrin and thiodan on the testicular histomorphology and histochemistry of earthworm Eudichogaster kinneari. Therefore the present work aims to show clearly the changes produced after exposure of safe concentration of dimethoate (0.6 ppm), azodrin (0.5 ppm) and thiodan (0.003 ppm) for twenty days in the testis of an earthworm Eudichogaster kinneari to evaluate histomorphological and histochemical abnormalities in their testis.

MATERIAL AND METHOD :

Healthy and sexually matured specimens of Eudichogaster kinneari approximately of same weight (6.5 + 0.001 gm), length (80-120mm) and diameter (5-7 mm) were collected from the vicinity of Ujjain city, India and acclimated in the laboratory in culture pots with moistened soil, before the commencement of the experiment. 40 earthworms were kept in each pot which was filled with 9000 gm soil. The earthworms were fed with organic matter, such as decaying leaves, compost manure etc.

The market sample of Dimethoate (Rogor 30E Rallis, India Ltd), Azodrin (monochrotophos, “Nuvacron” shell development co.) and Thiodan (Endosulfan, Southern minerals limited Haryana) were used for

experimental purposes, Dimethoate and Azodrin are organophosphorous and Thiodan is organochlorine insecticide.

Lc-50 value of these insecticides for Eudichogaster kinneari was determined. The calculated quantity of dimethoate, azodrin and thiodan was taken and diluted to 500 ml with tap water for preparation of the 0.6 ppm test concentration for dimethoate, 0.5 ppm concentration for azodrin and 0.003 ppm concentration for thiodan.

The prepared solution was sprayed on soil and mix with soil properly on the first day and on the 10th day of experiment. The control worms were kept in the soil without addition of insecticide. Both control and experimental animals were kept in identical conditions and the experiment was continued for 20 days and the organs were fixed in fixatives after 10 and 20 days. Before making the histological and histochemical preparations, the worms were narcotized and the organs were immersed in saline solution (0.75%) for a few minutes to avoid contractions. The testes were fixed in aqueous Bouin's fluid and 10% formalin. The fixed testes were processed for dehydration and blocks were prepared in paraffin wax, sections were cut at 4-5 µm and stained with Delafield's Haematoxylin and Eosin and Mallory's triple for histological details and Periodic Acid Schiff's (PAS),Mercuric Bromophenol Blue (Hg-BPB), Luxol Fast (LF) ,Best Carmine (BC) and Sudan Black B (SBB) for histochemical details. Statistical analysis of data was carried out by student's't' test.

RESULTS AND DISCUSSION :

CONTROL GROUP:

There are two pairs of testes, one on each side of the ventral nerve cord in the 10^{th} and 11^{th} segments. These are creamish or whitish in color, each testis is attached at its basal end to the septum while the rest part

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is protected by thread like ligaments, the testes are free and are not enclosed in a testis sac.

The spermatic follicles of testis of E.kinneari were arbitrarily classified into four consecutive developmental stages, depending on the size of spermatic follicles and approximate number of cells per cluster.

Stage 1: Immature spermatic follicles: Included small clusters having approximately 1 to 16 cells or fewer cells and measured 29.22+1.2µ. Cells joined together by a small central Cytoplasmic bridge, the cytophore. The cells are rounded and contained abundant cytoplasm (fig. 1 and 2).

Stage 2: Premature spermatic follicles: Included larger clusters with approximately 32-64 cells and measured 39.0±1.7µ. The developing sperm cells are larger and rounded with more prominent cytoplasm and nucleus (fig. 1 and 2).

Stage 3: Maturing spermatic follicles: Included larger clusters having approximately 64-128 cells and measured 56.75±1.7µ. The developing sperm cells are small, elliptical having a very prominent and much bigger cytophore. The signs of development of sperm tail are evident in some spermatic follicles (fig. 1 and 2).

Stage 4: Fully mature spermatic follicles: Spermatic follicles showed further development compared to those of stage-III, having approximately 128 cells and measured 60.37±1.6µ.The cytophore was larger still having a distinct freely moving sperm tail and the heads attached with cytophore (fig.1 and 2).

Histochemically all spermatic follicles showed mild reactions with periodic acid Schiff's (PAS) technique, all spermatic follicles showed mild reactions which suggest the presence of least quantity of carbohydrates. Mercuric Bromophenol blue (Hg-BPB) test revealed moderately positive results, indicating the presence of proteins. Lipids have been traced in minute quantities and phospholipids in sufficient quantities evidenced by

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Sudan black B (SBB) and Luxol fast (LF) techniques. Presence of glycogen was also observed in less quantity with Best Carmine (BC).Table 1.

TREATED GROUP:

10 DAYS EXPOSURE:

Exposure of E.kinneari to dimethoate for 10 days showed vacuolization in spermatic follicles (Fig.3).

Azodrin treated spermatic follicles showed dissolution at many places, cells and cytophore of spermatic follicles exhibited vacuolization (fig.4). Thiodan treatment depicted vacuolization due to shrinkage of spermatogenic components, thickened lining of spermatic follicles were also noticed (fig.5).

Decreased intensity with histochemical reactions (Table 1) and significantly reduced diameter of spermatic follicles (Table 2) and (fig.10) were seen.

20 DAYS EXPOSURE:

20 days treatment of dimethoate treated spermatic follicles showed shrinkage, granulation and vacuolization in cytoplasm of their cells (Fig.7).

Azodrin treatment showed broken spermatic follicles, due to which cells scattered everywhere within the sac. Almost all follicles showed clumping, vacuolization in their cells and in their cytophore, ultimately cellular architecture showed atrophied condition (Fig.8).

20 days thiodan treatment showed drastic effects in all spermatic follicles caused complete degeneration of tissues with broken follicles, destruction of cellular architecture caused by necrosis, lesions and congregation of spermatogenic material (Fig.9). Histochemically all stages of spermatic follicles exhibited less intensity (Table-1) and significantly

reduced size of ($p<0.001$) spermatic follicles were seen (Table-2) and (fig.10) when exposed with above insecticides.

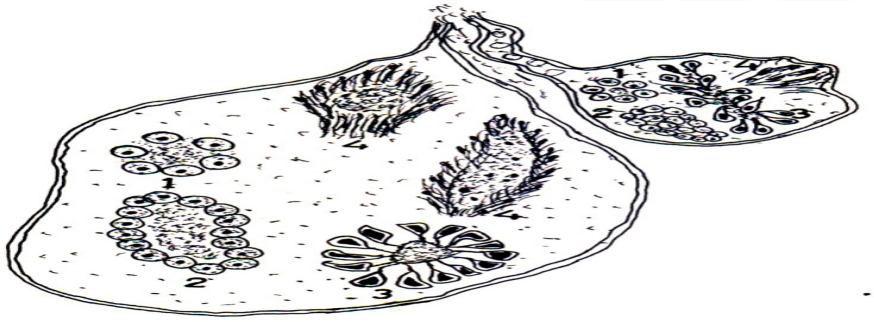

FIGURE 1

PHOTOGRAPH OF T.S. MALE GONAD OF E. KINNEARI SHOWING DIFFERENT STAGES OF SPERMATOGENESIS

1. Immature spermatic follicles
2. Premature spermatic follicles
3. Maturing spermatic follicles
4. Fully mature spermatic follicles

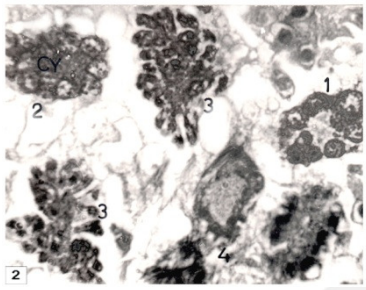

FIGURE 2

10 DAYS T.S. CONTROL TESTIS

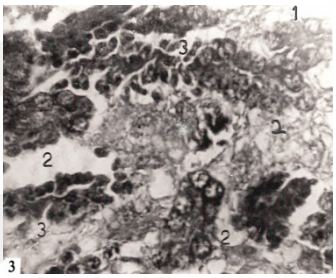

FIGURE 3

10 DAYS T.S DIMETHOATE TREATED TESTIS

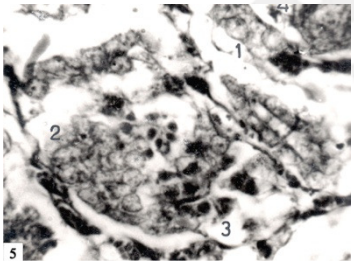

FIGURE 4

10 DAYS T.S AZODRIN TREATED TESTIS

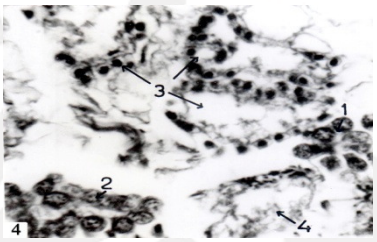

FIGURE 5

10 DAYS T.S. THIODAN TREATED TESTIS

1. Immature spermatic follicles
2. Premature spermatic follicles
3. Maturing spermatic follicles
4. Fully mature spermatic follicles

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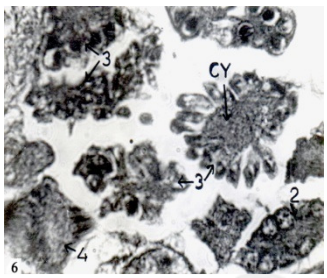

FIGURE 6
20 DAYD CONTROL TESTIS

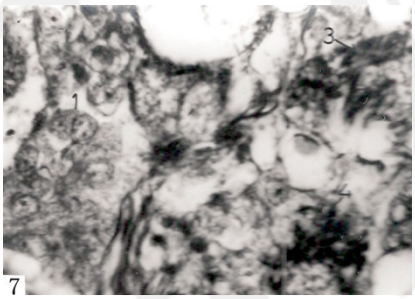

FIGURE 7
20 DAYS T.S. DIMETHOATE TREATED TESTIS

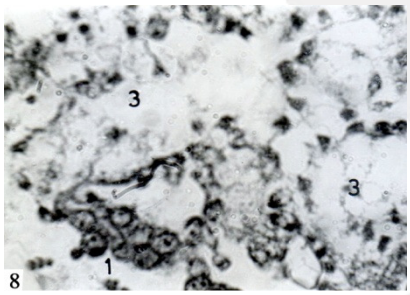

FIGURE 8
20 DAYS T.S. AZODRIN TREATED TESTIS

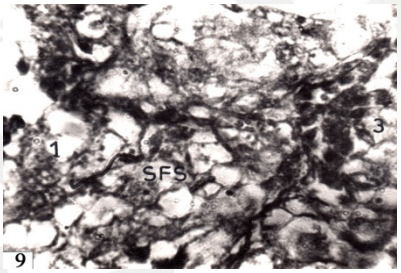

FIGURE 9
20 DAYS T.S.THIODAN TREATED TESTIS

1. Immature spermatic follicles 2. Premature spermatic follicles
3. Maturing spermatic follicles 4. Fully mature spermatic follicles

TABLE 1: HISTOCHEMISTRY OF TESTIS OF E. KINNEARI EXPOSED WITH INSECTICIDES

Days of treatment	Treatment	Sublethal concentrations used	Histochemical Test				
			PAS	Hg-BPB	LF	SBB	BC
10 Days	Control		++	+++	+++	++	+++
	Dimethoate	0.6 ppm	++	+++	+++	++	+++
	Azodrin	0.5 ppm	++	+++	+++	++	+++
	Thiodan	0.003ppm	++	+++	+++	++	+++
20 Days	Control		++	+++	+++	++	+++
	Dimethoate	0.6 ppm	+	+	+	+	+
	Azodrin	0.5 PPM	±	+	+	±	+
	Thiodan	0.003 ppm	±	+	+	±	+

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PAS-Periodic Acid Schiff's, Hg-BPB- Mercuric Bromophenol blue, LF- Luxol Fast, SBB- Sudan black B, BC-Best Carmine +++,++ Positive reactions, + Mild Positive reactions, ± Not clear

TABLE 2: DIAMETER OF SPERMATIC FOLLICLES OF E. KINNEARI EXPOSED WITH INSECTICIDES

Days of treatment	Treatment	Sublethal concentrations used	Diameter of Spermatic Follicles			
			Stage 1	Stage 2	Stage 3	Stage 4
10 Days	Control	-	29.05±1.4	38.9±1.1	56.25±1.0	60.5±1.8
	Dimethoate	0.6 ppm	22.25±1.5*** -(24.5)	33.87±1.0*** -(29.9)	51.87±1.6*** -(7.7)	51.5±1.9*** -(14.8)
	Azodrin	0.5 ppm	21.5±1.6*** -(27.1)	32.75±1.8*** -(15.8)	50.87±1.2*** -(9.5)	51.5±1.9*** -(14.8)
	Thiodan	0.003ppm	17.24±1.6*** -(41.5)	29.5±1.2*** -(24.1)	47.25±1.2*** -(19.0)	44.75±1.6*** -(26)
20 Days	Control	-	29.12±1.2	40.25±1.7	56.75±1.7	59.5±1.4
	Dimethoate	0.6 ppm	18.87±1.7*** -(35)	29.5±1.2*** -(26)	47.25±1.2*** -(16.7)	45.87±1.1*** -(22.9)
	Azodrin	0.5 ppm	17.24±1.6*** -(40.7)	28.22±1.2*** -(29.8)	45.12±1.8*** -(20.0)	45.37±2.6*** -(23.74)
	Thiodan	0.003ppm	12.75±1.3*** -(56.2)	21.47±1.8*** -(46.6)	40.62±1.5*** -(28.42)	37.0±1.8*** -(37.8)

All Values are expressed as mean+ SD: No.=10 Significant levels ***,**,*****. Values in parenthesis are % alterations

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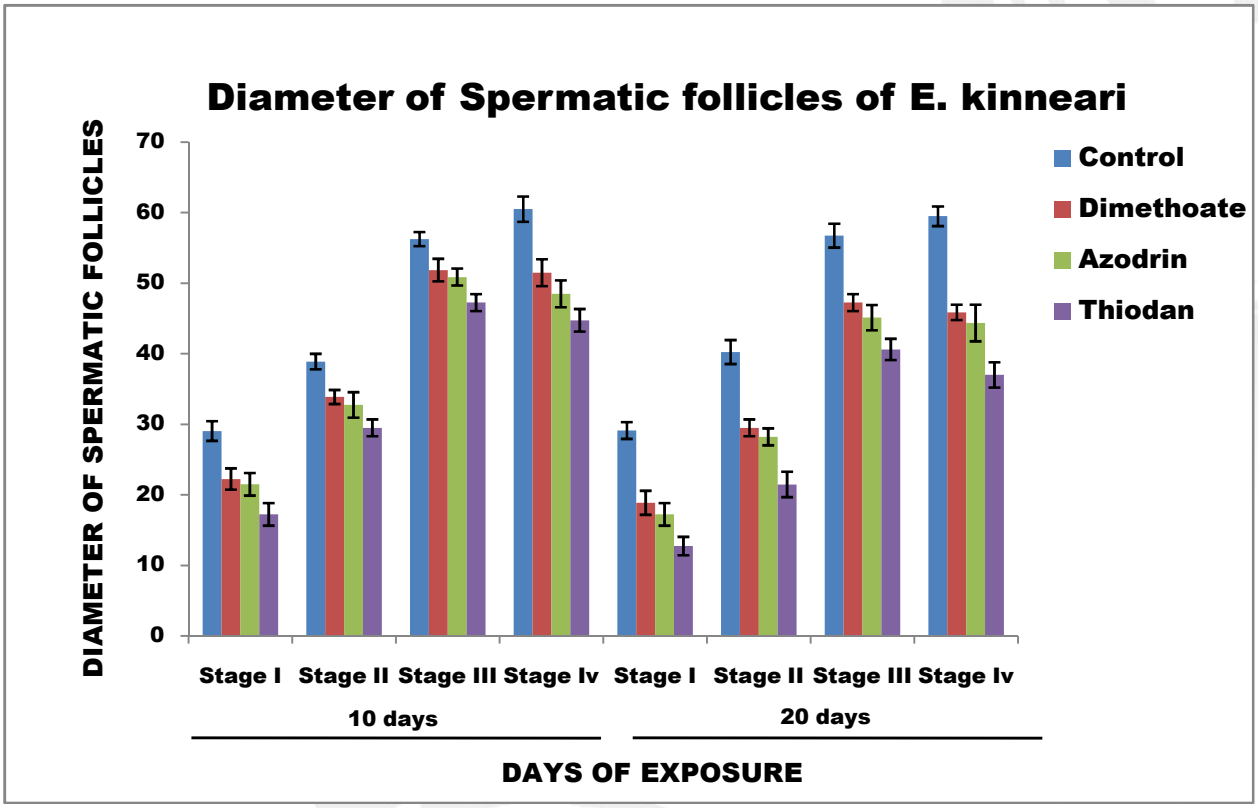

FIGURE 10

Numerous reproductive parameters have been studied in earthworms, exposed to various insecticides and chemicals viz: cocoon production, a reduced mean and maximum number of hatchlings per cocoon, sperm production, cytotoxicity, generotoxicity. Scientist have reported that pesticides influence the reproduction of worms in a dose dependent manner with greater impact of higher concentration of chemicals (Espinoza and Bustos 2004, Rao 2004, Calisi et al. 2009, De Silva et al. 2010, Gupta et al. 2010, Yasmin and D'Souza 2010, Damm et al. 2011 , Santos et al. 2012, Wang et al. 2012, Ahmed 2013, Pelosi et al. 2013 and Celine et al. 2014) but there is no report regarding the effect of these three insecticides i.e. dimethoate, azodrin and thiodan on histopathology of testis of earthworms E.kinneari.

The present investigation revealed that dimethoate, azodrin and thiodan at 0.6 ppm 0.5 ppm and 0.003 ppm concentrations respectively, for 20

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days exposure, arrest testicular functions in E.kinneari. Cytoplasmic and nuclear abnormalities were also observed in all stages of spermatic follicles. The cellular architecture of all stages of spermatic follicles were severally destructed, follicles lost their normal shape, asymmetrical arrangement of spermatozoa around the cytophore were seen, vacuolization, clumping of spermatogenic material, broken spermatic follicles and less intensity with histochemical reactions were noticed. The important observations were noticed with exposure of above three insecticides, was that the destruction was seen with thiodan exposure was more severe than azodrin and dimethoate. Thiodan exposure also showed cytotoxic effect caused by coiling of tail, sluggish movement and discharge of coelomic fluid.

Similar results were reported by Lakhani et al. (1991), Lakhani and Khatri (2008) and Lakhani (2007) in ovaries of Eudichogaster kinneari, when treated with above used three insecticides at same concentration and duration and found impaired ovarian functions, destruction in cellular architecture, less intensity with histochemical reaction and significantly decreased size of oocytes of different stages. Cikutovic et al. (1993) noted significant reduction in spermatozoa from testes and seminal vesicles after exposure with chlordane (6.25, 12.5 and 25 ppm) and cadmium nitrate (100, 200 and 300 ppm) in Lumbricus terrestris. Espinozoa-Navaroo et al. (2004) observed decreased viability of sperms in spermathica and cytotoxic effect by coiling of tail produced by malathion in Eisenia fetida.

Yasmin and D'souza (2010) studied on Eisenia fetida and observed impaired growth and reproduction produced by carbendazin, dimethoate and glyophosate and reported severe results by carbendazin and dimethoate than glyophosate. De Silva et al. (2010) observed decreased reproduction in Perionyx excavatus when treated with formulated carbafuran and decreased toxicity of three chemicals in order of

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carbafuran > chlorpyriphos > mancozeb. Gupta and Saxena (2010) reported sperms head abnormalities produced by carbaryl at 0.125 mg/Kg, amorphous sperm head at 0.25 mg/Kg and granulated nucleus of sperm head at 0.5 mg/Kg concentrations of carbaryl in testis of Metaphire posthuma. Ahmed (2013) reported toxicity symptoms in Lumbricus terrestris such as swollen body, sluggish movements , discharge of coelomic fluid and decrease total sperm numbers when treated with four pesticides (cyren,ridomil,triplen and mamba) and noted most toxic effects with cyren, moderately toxic with triplen and mamba and least toxic with ridomil.

Kale and Krishnamurthy (1982) in Pontoscolex corethurus, Sagar (1989) in Poecilobdella granulosa Rao (2004) in Eisenia fetida and Calisi et al. (2009) in Eisenia fetida studied acetyl cholinesterase activity with the effect of different insecticides and noted significantly decreased enzymatic activity, which in turn affect the process of gametogenesis as regulated by the gonadotropic hormones in the brain of annelids.

CONCLUSION :

Based on the observations of the present study and previous studies, it can be concluded that the reproductive parameters of earthworms affected by above insecticides seems to be useful bioindicators of soil pollution and indicate negative impact of pesticides on earthworm's reproduction. It is expected that when the earthworms E.kinneari were exposed to above three insecticides for 20 days at safe concentrations, their cellular enzyme system might have been disturbed, as the disturbed nervous system might have been affected the release of gonadotropins, which are essential for gametogenesis in E.kinneari.

As we know that earthworms are old friends of farmers, it is necessary to minimize the after effects of insecticides in agricultural fields as to save the earthworms. Application of insecticides should be restricted to needed places only, especially during breeding time when the

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earthworms are near to soil surface. The products which are used in agriculture fields should be least injurious to earthworms.

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