



Effect of Three Commonly Used Insecticides on Histomorphology and Histochemistry of ovary of an Earthworm *Eudichogaster kinneari* (Stephenson)

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Abstract

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 20 days to evaluate profound changes in the histomorphology and histochemistry of ovary by adding vacuolization due to congregation of ooplasm and nucleoplasm, irregular thickened cell boundary of oolema and nucleolemma, uneven stain were seen in whole structure, deterioration of ooplasm and nucleoplasm and ultimately destruction of cellular architecture of all stages of oocytes showed atrophied condition. Decreased intensity with histochemical reactions and reduced diameter of all stages of oocytes ($p < 0.001$) were observed. The present study indicates that among the three insecticides tested, thiodan 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.

Keywords: *Eudichogaster kinneari*, Insecticide, Ovary, Histomorphology, Histochemistry.

Introduction

The earthworm is one of the “nature’s top soil scientists” which make our soil good enough to grow healthy plants and provide us food. Earthworms decomposes plant residues, they break organic matter and leave behind castings that are a very valuable type of fertilizer, for this they bring down organic matter from the top layer of soil and mixing it with the soil below by involving ingestion, digestion and absorption of castings through the worms metabolic system¹⁻².

Charles Darwin³ referred to earthworms as “nature’s ploughs” because mixing of soil and organic matter. This mixing improves the soil fertility and the nutrients held in it to become available to bacteria, fungi and plants. Besides this, earthworms also maintain physical soil characteristics such as aeration, water permeability and mineral turnover by making both horizontal and vertical burrows in the soil along with their castings and body secretions; therefore they have been called ecosystem engineers”.

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 pollutants⁴⁻⁷. 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 agro chemicals not only affect the insects but equally damage the soil fauna. The effect

of pesticides on the reproductive organs of some invertebrates has been investigated^{8,9}. In spite of this, there is lack of information on the effect of three commonly used insecticides dimethoate, azodrin and thiodan on the ovarian histomorphology and histochemistry of earthworm. Therefore the present work aims to show clearly the changes produced after exposure of safe concentration of dimethoate (0.6ppm), azodrin (0.5ppm) and thiodan (0.003ppm) for 20 days in the ovary of an earthworm *Eudichogaster kinneari* to evaluate histomorphological and histochemical abnormalities in their ovaries.

Material and Methods

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 fixative after 10th and 20th days. Before making the histological preparations, the worms were narcotized and the organs were immersed in saline solution (0.75%) for a few minutes to avoid contractions. The ovaries were fixed in aqueous Bouin's fluid and 10% formalin. The fixed ovaries 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 ovaries, one on each side of the ventral nerve cord in the 13th segment. These are creamish or whitish in colour. Each ovary is attached at its basal end to the septum, while the free end floats in the coelom. Each ovary measured about 880 μm in length and 250 μm in width. The basal part of the ovary contains undifferentiated rounded cells, which are followed by a zone of dividing cells while their distal lobulated processes are longer and have ova of various developing stages which are present in linear arrangement, like undifferentiated, than differentiated, previtellogenic and the vitellogenic oocytes. Their arrangement starts from ovarian cord towards periphery, figure-1.

During Oogenesis changes are noticed in shape, size and organization of oocytes. Four different succeeding stages have been noticed in oogenesis.

Undifferentiated oocytes: These are the youngest oocytes, either round or oval in shape, measuring 0-15 μm in size with homogenous ooplasm. These are present in the ovarian cord and are not differentiable, prospective ova not noticeable different from the remaining cells when stained with heamatoxylin-eosin, figure-1, 2, and 3.

Differentiated oocytes: These are bigger in size than the stage-I oocytes. These are spherical to oval in shape. Each oocyte has a single large nucleus and homogenous ooplasm. The diameters of these oocytes measures 16 μm -31 μm . staining characteristics

are similar to those of other cells, figure-1 and 2.

Previtellogenic oocytes: These are much larger than stage I and stage II oocytes of germinal cord, measures from 32 μm - 64 μm or larger. These are spheroid or round in shape, having a single nucleus with nucleolus and homogeneous ooplasm. There is no sign of yolk accumulation. Staining blue with haematoxylin-eosin. The nucleoplasm with some granular patches of nuclear origin is distinguishable, figure-1, 2 and 3.

Vitellogenic oocytes: These are the largest oocytes, measuring 65 μm - 93 μm or larger, which are mostly spherical in shape. These oocytes show transitional stages from the beginning of yolk accumulation to the mature ovum. Yolk brilliantly stains with heamatoxylin having very clear nucleus (Fig.1 and 3). Sometimes two or more nuclei are seen in late vitellogenic stages (Fig.4). The mature oocytes are fully accumulated with yolk (Fig.5). Fully-grown mature oocytes are surrounded by a thin follicular membrane and are associated with a number of accessory cells derived from the protogonia of the presumption ovary, figure-6.

Histochemistry: With periodic acid Schiff's technique nucleus, nucleolus and ooplasm of all stages of oocytes showed mild positive reactions which suggest the presence of least quantity of carbohydrates and with Alcian blue showed negative reactions, which signifies absence of acid mucopolysaccharides. Mercuric Bromophenol blue test (Hg-BPB) revealed moderate positive results, indicating the presence of sufficient quantity of proteins. Lipids and phospholipids also have been traced in sufficient quantities as evidenced by Sudan Black B (SBB) and Luxol Fast (LF) techniques. Presence of glycogen was also observed in less quantity with Best Carmine (BC), Table- 1.

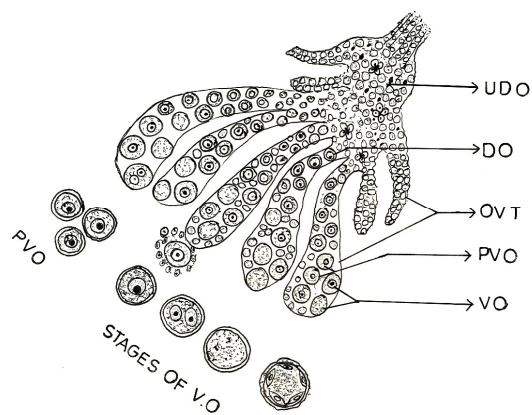


Figure-1

Photomicrograph of T.S. of Female gonad of *Eudichogaster kinneari* showing different stages of oocytes
OVT: Ovarian Tubule, UDO: Undifferentiated oocytes,
DO: Differentiated oocytes, PVO: Previtellogenic oocytes,
VO: vitellogenic oocytes.

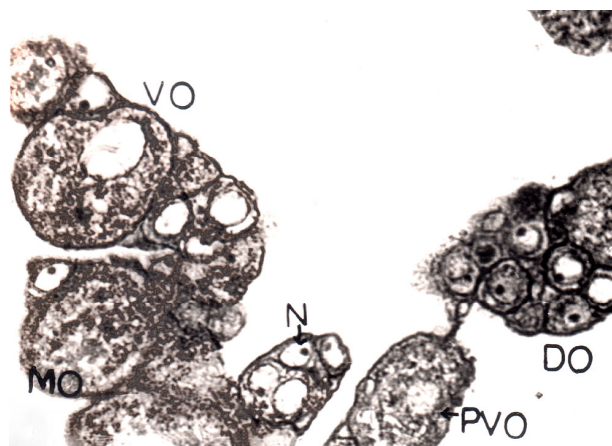


Figure-2

T.S. Female gonad of E.kinneari showing different stages of oocytes

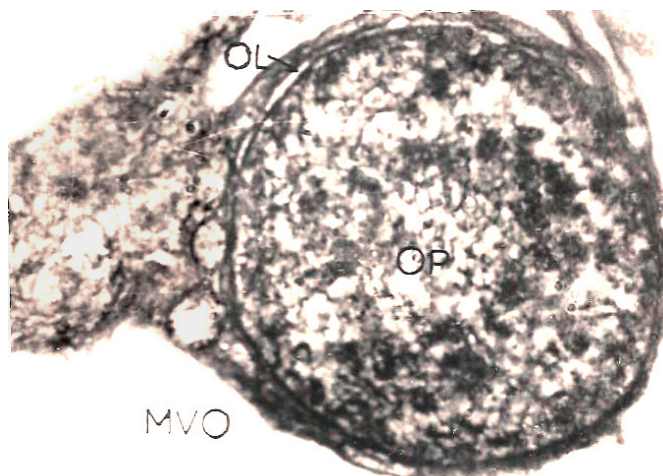


Figure-5

T.S. Female gonad of E.kinneari showing Mature Vitellogenic Oocyte

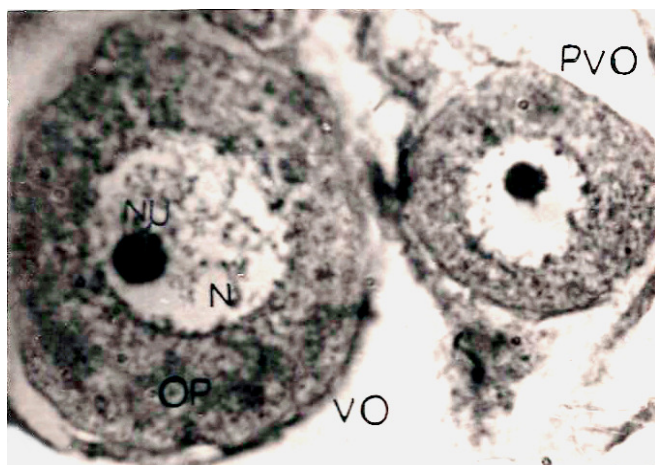


Figure-3

T.S. Female gonad of E.kinneari showing Previtellogenic and Vitellogenic Oocytes

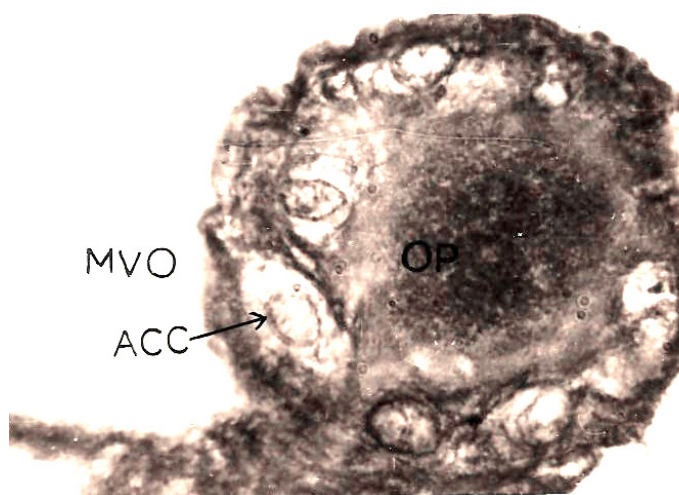


Figure-6

T.S. Female gonad of E.kinneari showing fully grown mature Oocyte with accessory cells

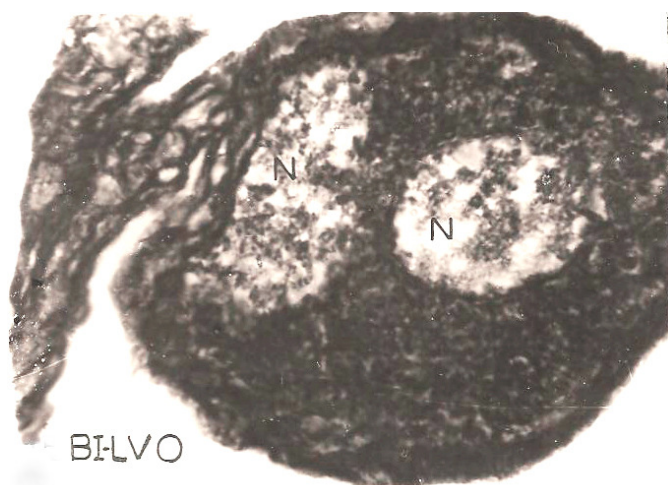


Figure-4

T.S. Female gonad of E.kinneari showing Binucleated Vitellogenic Oocytes

Treated Group: 10 Days Exposure: Exposure of Eudichogaster kinneari to dimethoate for 10 days showed dissolved ooplasm due to which many vacuoles were formed. Figure-7 and 8.

Azodrin caused irregular thickened oolema and vacuolization of ooplasm at many places. Figure-9 and 10. Thiodan treatment showed separated oolema by rest part of oocyte due to congregation of ooplasmic material and vacuolization of ooplasm was also seen. Figure-11 and 12.

Histochemical reactions revealed less intensify (table- 1) and decreased diameter of oocytes of all stages with exposure of above three insecticides. (Table-2 and figure-19) were seen.

20 Days Exposure: Thickened oolema, irregular shape and

vacuolization of undifferentiated and differentiated oocytes were noticed in 20 days exposure of dimethoate. Separation of oolema due to congregation of ooplasm was seen in many mature oocytes. Figure-13 and 14.

20 days azodrin exposure caused irregular and empty structure of undifferentiated and differentiated oocytes, many oocytes exhibited vacuolization in their ooplasm, necrosis was observed in whole structure. Figure-15 and 16.

Thiodan treated oocytes of all stages showed deterioration of cellular architecture aided by lost of their normal structure, vacuolization, granulation and ultimately atrophy of whole structure. Figure-17 and 18.

Histochemically all stages of oocytes exhibited less intensity. Table-1 and significantly reduced ($P < 0.001$) diameter of oocytes of all stages (table- 2 and Figure-19) were seen.

Numerous reproductive parameters have been studied in earthworms exposed to various insecticides and chemicals: cocoon, hatching, sperms production, viability of the sperms produced, sexual maturation and generotoxicity and

cytotoxicity. Several scientists have reported that pesticides influence the reproduction of worms in a dose dependent manner with greater impact of higher concentration of chemicals¹⁰⁻²⁴.

The present investigation revealed that dimethoate, Azodrin and thiodan at 0.6ppm, 0.5 ppm and 0.003 ppm concentrations respectively for 20 days exposure, impaired ovarian functions. Nuclear and Cytoplasmic abnormalities were noticed in all stages of oocytes. The cellular architecture of all stages of oocytes was severely destructed. The ooplasm and nucleoplasm showed dissolution and vacuolization.

Due to vacuolization oocytes became empty or many empty spaces were seen in ooplasm. Oocytes lost their normal shape. Histochemical reactions depicted less intensity and decreased diameter of oocytes were noticed. The important observations were noticed with exposure of above three insecticides, was that the destructions were seen with thiodan exposure was more severe than azodrin and than dimethoate. Thiodan exposure also showed cytotoxic effects caused by coiling of tail, sluggish movement and discharge of coelomic fluid.

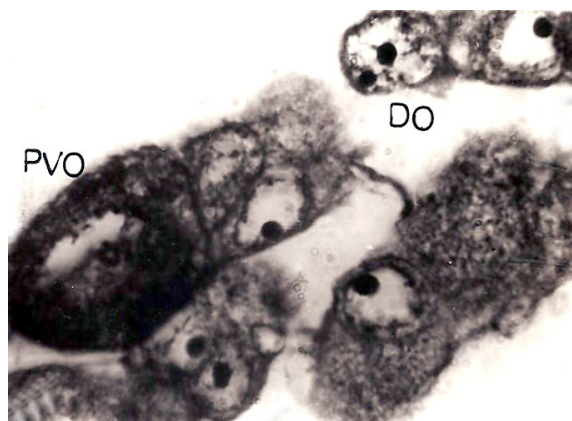


Figure-7
T.S. 10 days dimethoate treated ovary

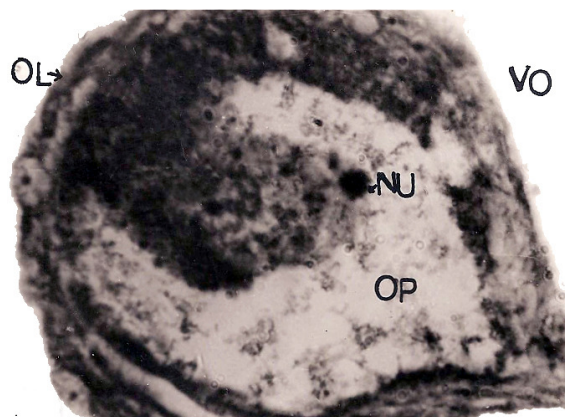
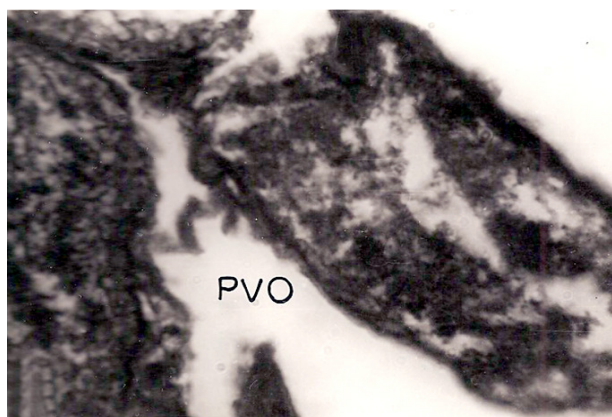
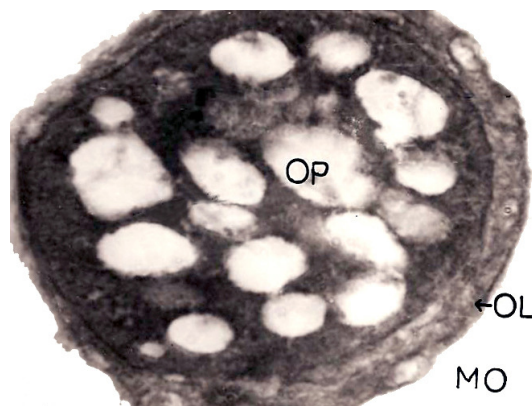


Figure-8
T.S. 10 days thiodan treated ovary

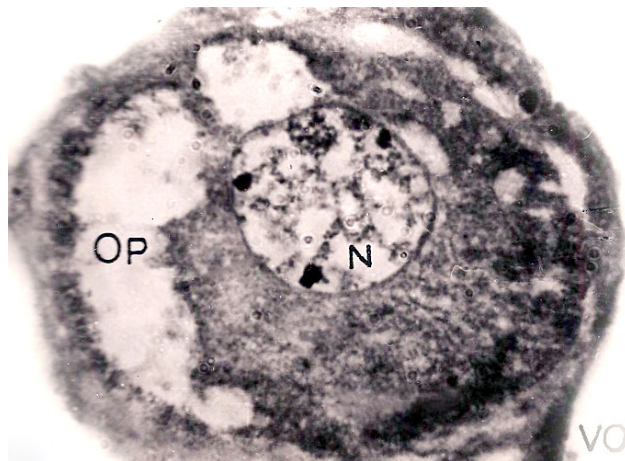
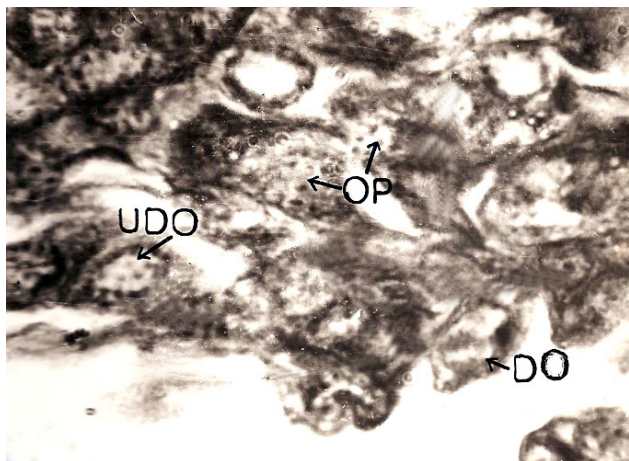


Figure-9
T.S. 10 days thiodan treated ovary

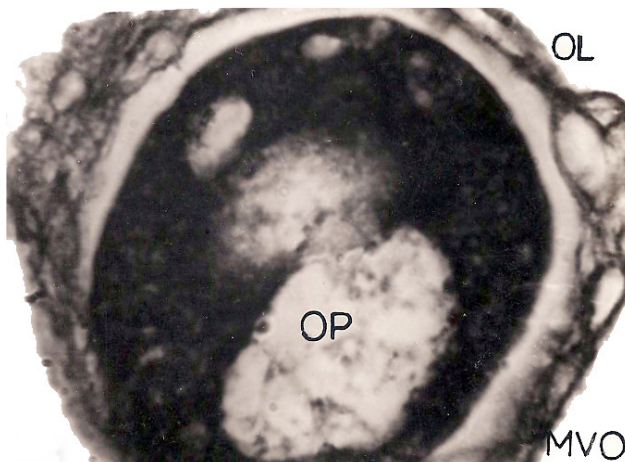


Figure-10
T.S. 20 days dimethoate treated ovary

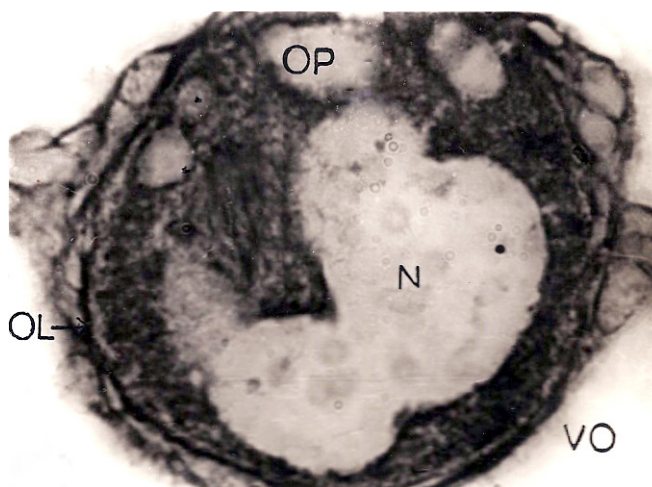
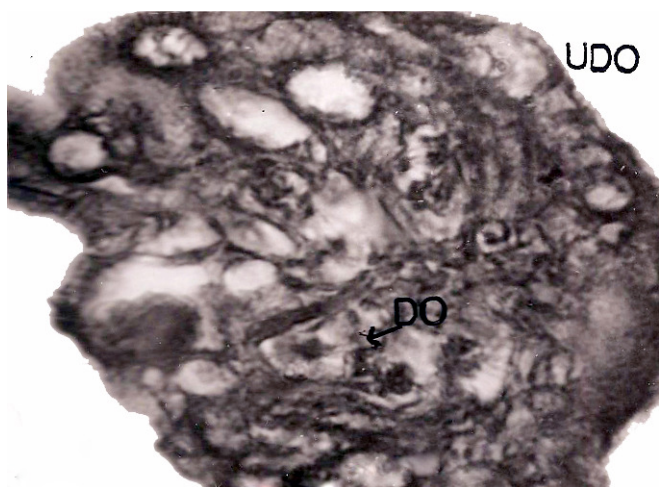


Figure-11
T.S. 20 days azodrin treated ovary

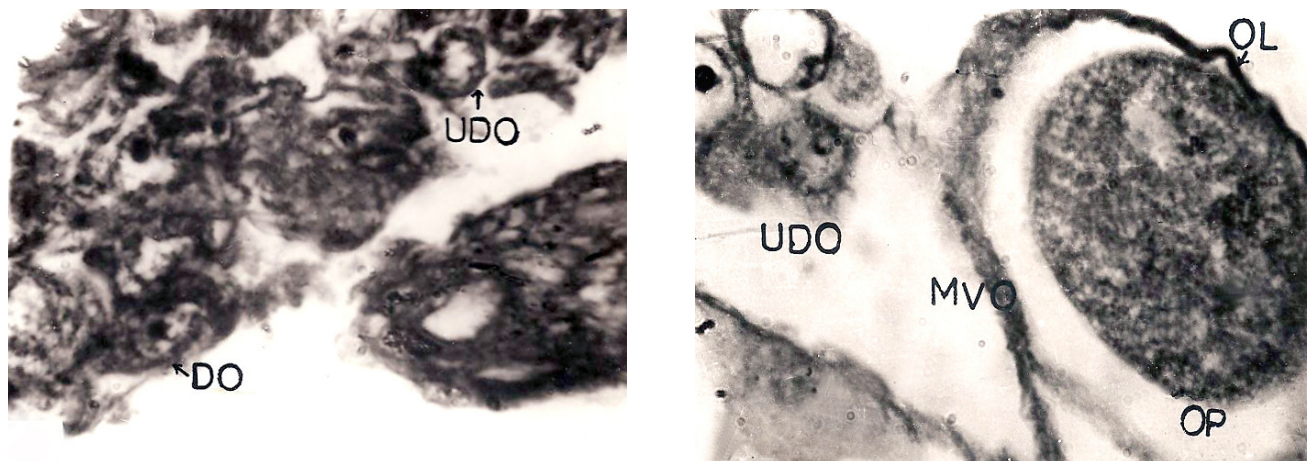


Figure-11
 T.S. 20 days thiodan treated ovary

Table-1
 Histochemistry of ovary 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	±	+	+	±	+

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 Oocytes Of *E. kinneari* Exposed With Insecticides

Days of treatment	Treatment	Sublethal concentrations used	Diameter of oocytes			
			Stage 1	Stage 2	Stage 3	Stage 4
10 Days	Control	-	17.12±1.8	30.87±1.5	63.75±1.8	93.87±2.1
	Dimethoate	0.6 ppm	12.5±1.8*** -(26.9)	22.5±1.5*** -(27.1)	52.75±1.6*** -(17.2)	81.25±1.8*** -(13.4)
	Azodrin	0.5 ppm	10.25±1.7*** -(40.12)	20.0±1.8*** -(35.2)	49.25±1.8*** -(22.7)	76.17±2.4*** -(18.8)
	Thiodan	0.003ppm	9.5±1.7*** -(44)	16.75±1.4*** -(45)	46.62±1.4*** -(26.8)	71.75±1.4*** -(23.5)
20 Days	Control	-	16.25±1.8	31.0±.98	63.62±1.9	94.62±1.6
	Dimethoate	0.6 ppm	8.5±1.6*** -(47.6)	14.19±1.8*** -(54.2)	40.62±1.5*** -(36.15)	68.75±1.8*** -(27.3)
	Azodrin	0.5 ppm	8.07±1.3*** -(50.3)	12.44±1.3*** -(59.8)	39.0±1.8*** -(38.6)	58.0±1.5*** -(38.7)
	Thiodan	0.003ppm	6.37±1.0*** -(60.8)	9.5±1.7*** -(69)	33.87±1.0*** -(46.7)	54.87±1.8*** -(42)

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

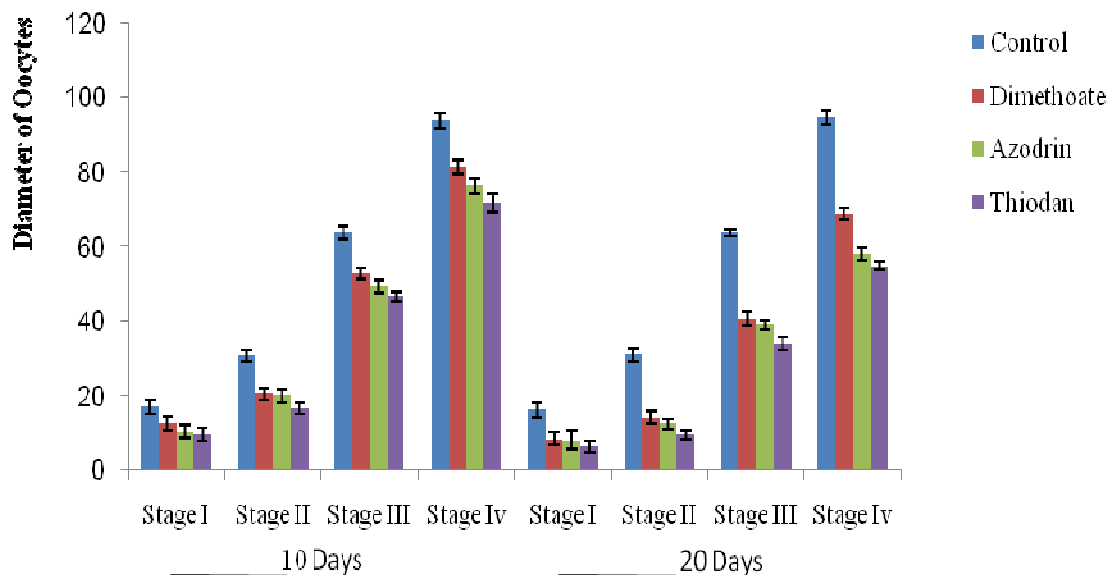


Figure- 12
Days of Exposure

Similar results were reported viz. ceasing of testicular maturation and atrophy in cellular architecture of spermatogenic follicles in testis of *Eudichogaster kinneari*, loss of normal shape of spermatogenic follicles, asymmetrical arrangement of spermatozoa around the cytophore, vacuolization, congregation of spermatogenic material, less intensity with histochemical reactions and reduced diameter of spermatogenic follicles in all stages ($p < 0.001$) when treated with above used three insecticides at same concentrations and duration¹¹⁻¹³. Similar results were observed in ovary of *Hirudo birmanica* when exposed with endosulfan, malathion and CuSO_4 at different concentrations for 20 days, arrest of ovarian cord caused by endosulfan treatment, while decreased number of differentiated and previtellogenic oocytes with malathion treatment and ceasing of ovarian maturation caused with CuSO_4 treatment⁸. Same observations were also noticed in gonads of *Poecilobdella granulosa* with treatment of endosulfan, malathion and sevin⁹. Decrease viability of sperms in spermatheca and cytotoxic effect by coiling of tail produced by malathion in *Eisenia fetida*¹⁴. Impaired growth and reproduction produced by carbendazim, dimethoate and glyphosate and reported severe results by carbendazim and dimethoate than glyphosate in *Eisenia fetida*¹⁵. Sperm 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*¹⁶. Decreased reproduction was noticed in *Perionyx excavatus* when treated with formulated carbofuran and toxicity of three chemicals in the order carbofuran > chlorpyrifos > mancozeb were observed¹⁸.

The insecticide endosulfan, Malathion and sevin decreased activity of enzyme acetylcholinesterase in the nervous system of *Poecilobdella granulosa*⁹. The activity of this enzyme also

decreased in *Pontoscolex corethurus* when treated with sevin¹⁹ and in *Eisenia fetida* when treated with azodrin²⁰.

Conclusion

On the basis of above findings, it is concluded that profound changes in the ovaries of *Eudichogaster kinneari* after treatment with above insecticides are produced. Affected organs of earthworm seem to be useful bioindicators of soil pollution and indicate negative impact of pesticides on earthworm's reproduction. The *Eudichogaster kinneari* were exposed with above insecticides, their cellular enzyme system have been disturbed. The disturbed nervous system might have been affected the release of gonadotropins, which are essential for normal process of gametogenesis in *Eudichogaster kinneari*.

As we know the importance of earthworms in agricultural fields and in animal feed industries, 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 and in rainy season when the earthworms are near to soil surface. The products which are used in agriculture fields should be least injurious to earthworms.

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