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Ovarian Histological Analysis of *Anabas testudineus* as a Marker for Pesticide Residue Analysis in Freshwater Ecosystems

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Abstract

In response to the increasing need for sustenance for the global human population, agricultural areas have witnessed the widespread application of pesticides. However, this practice has led to the subsequent runoff to the neighbouring water bodies, ultimately contributing to the degradation of ecosystems. The primary objective of this study is to examine the detrimental impacts on the histological structure of the ovary in *Anabas testudineus* fish species when exposed to both the 96-hour Lethal Concentration (LC_{50}) and sublethal concentration of endosulfan (one-tenth of 96-hour LC_{50}) over 30 days. After the exposure period, live fish were sacrificed and ovaries were dissected, pooled and fixed for routine histological procedures. After being subjected to the median lethal concentration for 96 hours, the pesticide-induced adverse alterations in the structure of the ovaries were studied. The ovaries of *A. testudineus* exhibited nuclear retraction, free-floating follicular lining, adhesion between ovarian follicles, and an elevated count of atretic oocytes following exposure to a sublethal dosage for 30 days. Histopathological investigations provide a helpful means for assessing the ecotoxicity of diverse chemicals, offering significant insights into the adverse effects resulting from the unwise and indiscriminate application of pesticides in agricultural settings. This study reveals the toxicity of organochlorine pesticide endosulfan on the female reproductive system of *A. testudineus* which directly affects the development and reproduction of fish.

Keywords: *A. testudineus,* Atretic Ovary, Endosulfan, Necrosis

1. Introduction

The contamination of aquatic ecosystems by pesticides poses a significant issue within the context of India. The introduction of these compounds into the environment might result in either immediate and evident harmful effects or more severe but less apparent impacts on non-target animals, such as fish, by compromising their long-term survival and reproductive capabilities. Fish are considered to be reliable bioindicators of habitat conditions and long-term toxic effects due to their mobility and comparatively extended life span[1]. The chlorinated hydrocarbon insecticide endosulfan belongs to the cyclodiene subgroup. It is classified as a broad-spectrum insecticide and has genotoxic, neurotoxic, haemotoxic, and nephrotoxic properties. Endosulfan exhibits limited solubility in water, resulting in its propensity to bioaccumulate through adsorption onto soil particles in groundwater, consequently leading to an elevation in its toxicological effects. The study on *Oreochromis mossambicus* exhibited modified oxygen equilibrium curves, a reduction in blood pH, and an

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elevation in CO_2 level following exposure to endosulfan[2]. Pesticides have a detrimental impact on lakes and rivers due to their extensive use, as they are carried into these water bodies by runoff from fields. Additionally, pesticides can accumulate in organisms and disrupt the food chain, which poses a threat to both animals and people[3,4]. Freshwater and marine ecosystems are crucial for the sustenance of various organisms and are responsible for upholding their overall well-being[5].

Endosulfan is a widely used pesticide that has been identified as an endocrine disruptor capable of interfering with the normal functioning of the reproductive system in female freshwater teleosts, such as *Anabas testudineus*. Exposure of *A. testudineus* to endosulfan resulted in significant histological abnormalities in the brain and spleen[6]. The analysis of gonadal tissue in freshwater bivalves after exposure revealed an elevation in glycogen concentration and a reduction in protein and lipid levels. This can be attributed to the organism's heightened reliance on metabolic and endocrine mechanisms to counteract the observed imbalance[7]. The overutilization of pesticides in agricultural and various food animal production systems leads to the contamination of adjacent natural water bodies, posing risks to aquatic organisms, particularly fish. These pollutants can be acquired by fish both directly from the contaminated water and indirectly through the food chain[8]. The study observed the aggregation and disintegration of ovarian tissues, as well as the breakdown of perinuclear ooplasm in fish, following exposure to carbofuran[9]. Reports indicate that certain teleost and zebrafish species possess metabolic characteristics that closely resemble those found in humans. This similarity makes them promising candidates for studying the cellular processes that occur in response to physical and chemical stimuli[1,10]. Histopathological examination is a helpful tool in the study of the impact of different chemicals and anthropogenic contaminants on aquatic organisms, providing insights into the overall consequences.

The health of the entire ecosystem is intricately connected to other biomarkers of stress because numerous contaminants require metabolic activation to induce cellular alterations inside the affected organism[11]. Water pollution has a broad impact on various aquatic organisms, with a specific emphasis on fish species. This ultimately has repercussions for human populations, since the contamination of food chains can lead to adverse effects[12]. Fish is a dietary source that is abundant in protein, carbohydrates, and various other essential nutrients. The health of fish will directly reflect the condition of the ecosystem, so ovarian histological analysis of *A. testudineus* exposed to various concentrations of endosulfan can be considered a marker for pesticide residue analysis in freshwater ecosystems. *Anabas testudineus,* commonly known as the climbing perch, is a resilient and palatable fish species found in Kerala, India. There is a lack of research in the Indian contest on ovarian histological assay of teleost as a biomarker for pesticide residue. So acute and sublethal toxicity of endosulfan on the female reproductive system was carried out to understand the extent of chemical pollution in the ecosystem. Our study aimed to analyse the ovarian histopathology of *Anabas testudineus* to varying doses of endosulfan.

2. Materials and Methods

2.1 Test Organism

The species *Anabas testudineus* is found in a diverse range of habitats, including lakes, marshes, rice fields, tributaries, and main river channels, across several regions such as India, Bangladesh, Pakistan, Sri Lanka, Southern China, and Southeast Asia. In certain geographical regions, the species is found in brackish coastal habitats, despite its primary habitat being lowland freshwater areas. The organism can survive without access to water for a duration of 6 to 10 hours, and if it retains moisture, it can traverse overland distances of several hundred meters. The propulsion of forward motion is facilitated by the lateral movements of the caudal fin and posterior region of the body. Stability is maintained through the extension of the pectoral fins and operculum, which possess robust and modified spines. The possession of supplementary respiratory organs allows it to withstand conditions of reduced oxygen levels. *Anabas testudineus*, commonly known as the climbing perch, is capable of undergoing aestivation for extended periods under harsh conditions by submerging itself in damp soil. The species in question is a consumable fish often found in Kerala, India.

2.2 Fish Collection and Acclimatization

Fish were taken at Akkulam Lake, located in Thiruvananthapuram, Kerala, India. A selection was made of exclusively robust fish specimens measuring 14-15 cm in length and weighing between 45-50 g. These chosen fish were subjected to a treatment involving

the application of a 0.05% solution of potassium permanganate for two minutes. The fish were placed in spacious glass aquaria, referred to as holding tanks, for two weeks to facilitate their adjustment to laboratory settings. During this period, the fish were exposed to seasoned tap water and provided with feed consisting of tapioca, groundnut oil cakes, rice bran, fish meal, and a sufficient quantity of vitamins. The fish were fed two to three times daily. Regular maintenance of the aquaria was conducted at 48-hour intervals to eliminate residual food particles and excretory waste.

2.3 Experiment Design

Before the experiment, the glass aquaria of identical dimensions underwent disinfection using Potassium Permanganate ($KMnO_4$) and were thereafter filled with 40 L of tap water that had been previously treated to remove chlorine. To establish control conditions, three glass aquaria were designated as blank solution tanks with an endosulfan concentration of 00 ppb. Additionally, three aquaria were allocated as experimental tanks for each concentration to determine the LC_{50}.

2.3.1 Water Quality Parameters

The details of water quality for the experiment were as follows

Temperature (25.32 ± 2.76 °C), pH (7.1 ± 0.09), dissolved oxygen (7.49 ± 0.58 mg/L) and free carbon dioxide (6.53 ± 1.02 mg/L).

2.3.2 Experiment Setup

The concentrations (3, 5, 7, 9, 14, 16, 18, 20, 25, 28, 31, 35, 38 and 40 parts per billion) of endosulfan was used for estimation of LC_{50}. Eight fishes in three replicates for each concentration (total 336 fishes). The participants refrained from consuming food for 24 hours before the commencement of the experiment, and this fasting practice was maintained throughout the study. The control and test solutions were renewed on a daily basis[13]. The sublethal concentration chosen for the current study was one-tenth of the 96-hour LC_{50}. A comparative study of ovarian histopathology of live fishes of 96h LC_{50} and sublethal concentration ($1/10^{th}$ of 96h LC_{50}) was carried out to analyse the extent of toxicity of chemicals. The fish specimens (eight fishes in three replicates) were subjected to a sublethal dose for 30 days, and the 96-hour LC_{50} (eight fishes in three replicates) for histopathological analysis.

2.4 Chemicals

The study utilized commercial-grade endosulfan (35 EC) that was procured from a local market. The experimental concentration of pesticide was created by conducting serial dilution of Commercial grade endosulfan (35 EC).

2.5 Histopathology

In the histological investigation, following the designated exposure period, the ovaries of both the control and treatment fish specimens were meticulously dissected, sectioned into smaller fragments, and subsequently immersed in Neutral Buffered Formalin (NBF) for two days. The fixed tissues were subsequently dehydrated using a series of ethyl alcohol solutions of increasing concentration until pure, anhydrous alcohol was achieved. Following dehydration, the tissues were cleaned using methyl benzoate and then embedded in paraffin. The tissue sections were prepared by cutting them at a thickness of 5 µm and thereafter stained with haematoxylin and eosin for histological observations.

3. Result

3.1 LC_{50} of Endosulfan for 24,48,72 and 96 h, Upper and Lower Confidence Limits

An acute static renewal test was performed for the determination of LC_{50} of endosulfan and was calculated as 27.58 ppb for 24 h with 31.37 ppb as the upper confidence limit and 24.24 ppb as the lower confidence limit. For 48 h LC_{50} of endosulfan was 23.35 and the upper and lower confidence limit was 24.57 ppb and 22.19 ppb. 18.79 ppb was LC_{50} for 72h with 19.17 ppb as the upper confidence limit and 18.42 ppb as the lower confidence limit. For 96 h LC_{50} of endosulfan was 16.91 ppb. The upper confidence limit was 18.08 ppb and the lower was 14.61 ppb. The sublethal concentration selected for the study was 1.69 ppb (One-tenth of 96h LC_{50}).

In teleost fish ovary has an outer thin peritoneum, inner to it is an ovarian wall which has outer tunica albuginea a thin connective tissue enveloped and an inner germinal epithelium composed of a single layer of cuboid cells. Smaller younger germinal cells, oogonia are grouped in oogonia nests between the more developed follicles. Oocytes in different development stages can be noticed in the ovary. Mature oocytes are larger than all other stages with many large yolk globules distributed throughout the cytoplasm.

3.1.1 Ovarian Histopathology of A. testudineus After Exposure to 96 h LC_{50} of Endosulfan

Histopathological studies of the female reproductive system of *A. testudineus* exposed to 96h LC_{50} and sublethal concentration for 30 days revealed serious damage to ovarian histological architecture. Necrosis (N), Vacuoles (V), Free-floating Follicular Lining (FFL) and Atretic Oocytes (AO) were observed when exposed to 96h LC_{50} of endosulfan (Figure 2). Control sections of the ovary were normal and without any histological alterations (Figure 1).

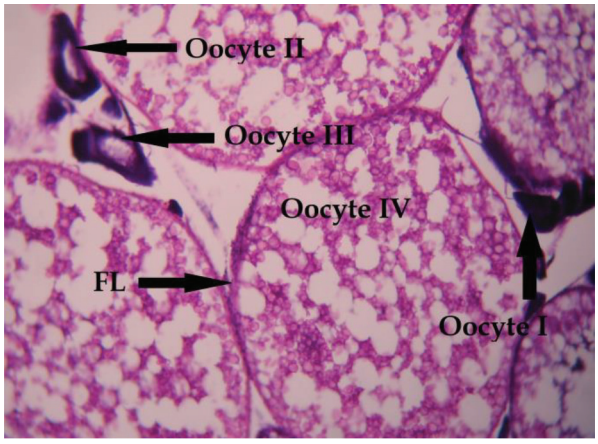


Figure 1. The control section of the ovary of *A. testudineus* with oocytes at various stages of development and without any histological alterations (for 96 hours).

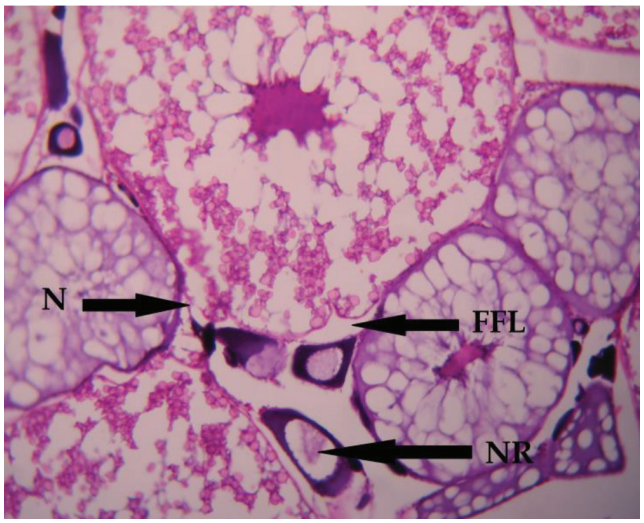


Figure 2. Ovary of *A. testudineus* exposed to 96h LC_{50} showed Necrosis (N), Vacuoles (V), Free-floating Follicular Lining (FFL) and Atretic Oocytes (AO).

3.1.2 Ovarian Histopathology of A. testudineus after Exposure to Sublethal Concentration of Endosulfan for 30 Days

Nuclear Retraction (NR), Free-floating Follicular Lining (FFL), adhesion between ovarian follicles (AD), an increase in the number of Atretic Oocytes (AO) and Damaged Stroma (DS) were observed in the ovaries of *A. testudineus* exposed to sublethal concentration for 30 days (Figure 3). Control sections of the ovary were normal without any histological alterations (Figure 4).

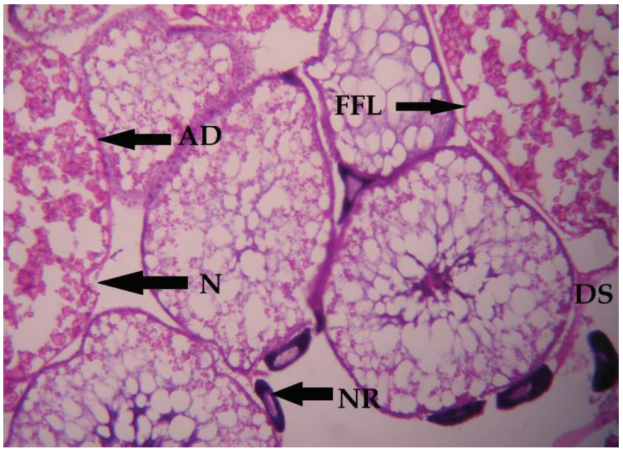


Figure 3. Ovary of *A. testudineus* exposed to sublethal concentration for 30 days showed Free-floating Follicular Lining (FFL), adhesion between ovarian follicles (AD), increase in the number of Atretic Oocytes (AO) and Damaged Stroma (DS).

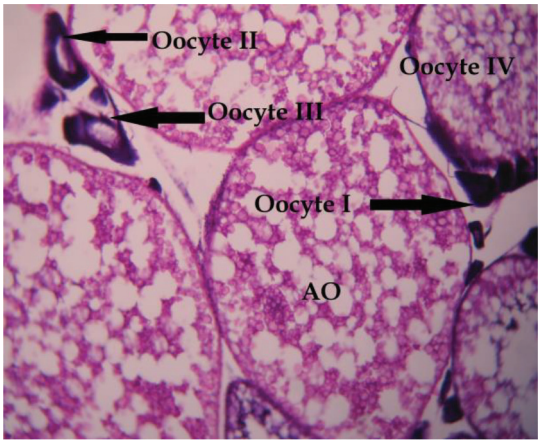


Figure 4. The control section of the ovary of *A. testudineus* with normal oocytes at various stages of development and Follicular Lining (FL) (for 30 days).

4. Discussion

The utilization of diverse agrochemicals in an imprudent, wide, and indiscriminate manner has been driven by the need to meet the escalating need for food due to population growth, the development of modern civilization, and current agricultural trends. The introduction of these chemicals into neighbouring water bodies has both direct and indirect consequences, resulting in an ecological imbalance. This imbalance adversely affects the essential organs of aquatic biota, with a specific impact on fish species. Ultimately, this disruption leads to a catastrophic outcome for the aquatic environment[14-18]. The entry of the organochlorine insecticide endosulfan into the creatures' bodies occurs through multiple pathways, including inhalation, ingestion, and dermal absorption. The potency of this chemical is such that even minute quantities of this pesticide within an organism's body can hinder or mimic the functions of hormones such as thyroid hormones, estrogen, and testosterone[19-23]. In the current investigation, the median lethal doses of endosulfan on *A. testudineus* were determined to be 27.58, 23.35, 18.79, and 16.91 parts per billion (ppb) for exposure periods of 24, 48, 72, and 96 hours, respectively. These findings unequivocally demonstrate a positive correlation between concentration and exposure duration, indicating an escalation in toxicity. In a study[24], the 96-hour LC_{50} of endosulfan for *Channapunctatus* was found to be 7.75 ppb. The observed LC_{50} values were found to differ among species due to variations in their ability to detoxify the compound, as well as the life cycle stage of the species. Additionally, the LC_{50} values were influenced by several elements present in the medium, such as hardness, turbidity, temperature, pH, and dissolved oxygen. The hydrolysis product of endosulfan, known as endosulfan sulfate, exhibits higher toxicity compared to endosulfan itself[25].

The current investigation involved an examination of the ovarian histology of *A. testudineus* following exposure to the LC_{50} 96 h concentration of endosulfan. The findings of this study indicated the presence of many abnormalities, including necrosis, vacuolization, free-floating follicular lining, and Atretic oocytes. Albino mice, upon exposure to endosulfan, exhibited the presence of atretic cells and experienced disruption in their oestrous cycle. This can be attributed to the direct impact of endosulfan on the hypothalamus-hypophysial-ovarian axis[26]. The toxicity of endosulfan disrupts the normal functioning of the reproductive system in female *A. testudineus*. Exposure to endosulfan has been found to have a significant impact on the female reproductive system of *A. testudineus*, a freshwater teleost. This disruption includes histopathological alterations in the ovaries and impaired production of vitellogenin. These effects can lead to reduced fertility, impaired follicular development, and disrupted oocyte development, ultimately impacting the population dynamics and sustainability of *A. testudineus* in freshwater ecosystems.

Various fishes exposed to pesticides exhibited irregular *Tunica albugenia*, ruptured ovigerous lamellae, damaged follicles, and atretic cells[27,28]. In a study conducted on freshwater fish species *Colisa fasciatus*, it was shown that exposure to a concentration of 1 ppm of endosulfan resulted in a retardation of ovarian activity and a significant reduction in the diameter of Stage II and Stage III oocytes[29]. A study found that exposure to endosulfan hurts the sexual attractiveness of female mosquitofish (*Gambusia affinis*)[30]. In our investigation, it was shown that *A. testudineus*, when subjected to a sublethal dosage for 30 days, exhibited various reproductive abnormalities. These included adhesion between ovarian follicles, nuclear retraction, free-floating follicular lining, and an elevated count of atretic oocytes. The impact of endocrine inhibitors, such as endosulfan, on fish fecundity is evident by the induction of atresia, leading to a decrease in reproductive capacity[26]. The male reproductive system of *A. testudineus* exhibited significant histological changes when subjected to endosulfan[31]. The ovary of bluegill sunfish that were subjected to endosulfan exhibited cytoplasmic retraction and nuclear retraction, as well as the raising of the ovarian wall and damage to the stroma[32].

5. Conclusion

The growing human population and modern civilization have led to the use of agrochemicals to boost crop production, causing ecological imbalance and affecting aquatic biota, particularly fish. The organochlorine pesticide endosulfan enters organisms through various routes, blocking or imitating hormones like thyroid hormones, estrogen, and testosterone. The lethal concentrations of endosulfan in *A. testudineus* were found to increase with concentration and exposure periods. The histology of the ovary of *A. testudineus* exposed to endosulfan showed abnormalities like necrosis, vacuolization, free-floating follicular lining, and atretic oocytes. This study reveals the toxicity of organochlorine pesticide endosulfan on

the female reproductive system of *A. testudineus* which directly affects the development and reproduction of fish. Reduced fecundity and abnormal offspring can result due to any endocrinological effects that the many pesticides of the world, including endosulfan, cause. The ovarian histopathology of *A. testudineus* may be considered as a biomarker of pesticide residue and chemical pollution of the ecosystem The study also points to the serious issue of how the indiscriminate use of pesticides in agriculture fields affects nontarget organisms and ecosystem stability.

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