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RESEARCH ARTICLE

STUDIES ON HISTOPATHOLOGICAL CHANGES IN THE GILL, LIVER, MUSCLE AND OVARY OF *OREOCHROMIS MOSSAMBICUS* (PETERS) EXPOSED TO DIISONONYL PHTHALATE (DINP)

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ABSTRACT

Diisononyl phthalate (DINP) is used as a plasticizer for PVC products. Freshwater fish, *Oreochromis mossambicus* were exposed to DINP at 300 ppm concentration for 24, 48, 72 and 96 h maintaining positive (propylene glycol as solvent) and negative (without solvent and toxicant) control groups. At the end of every treatment period, gill, liver, muscle and ovary were dissected and fixed in buffered formalin. Histopathological changes in gill includes epithelial lifting, hyperplasia, aneurysm, blood vessel dialation, lose of primary and secondary lamellae. DINP-induced hepatic damages like cytoplasmic vacuolization, spindle shaped nuclei and necrosis was observed. Muscle tissues undergone various types of damages such as thickening of muscle bundle, segmented muscle fibres and muscular dystrophy. Histopathological modifications in ovary were observed as progressive reduction in the number of matured oocytes, increase in distorted and atretic oocytes. The present study demonstrates that acute exposure to DINP alters the normal architecture of vital tissues in fish.

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INTRODUCTION

Diisononyl phthalate (DINP) belong to the class of dialkylphthalate esters represents a complex of branched predominantly C-9 isomers, and are used as plasticizers that makes polyvinyl chloride more flexible. DINP is also used in vinyl flooring, building materials, automobile interiors, medical devices, and childcare products as toys and footwears. Therefore, the routes of DINP exposure to humans occur mainly through oral, dermal and inhalation. The major source of childhood exposure to DINP is generally when the children mouth toys, where DINP and other plasticizers migrate into saliva and also dermally absorbed through the oral mucosa (Rastogi, 1998). The three major oxidative metabolites of DINP identified have been named as monoisononyl phthalates (MINPs) that includes, carboxy-MINP (CO_2-MINP), hydroxyl-MINP (OH-MINP) and oxo-MINP, which are then subjected to β-glucuronidation (Silva *et al.,* 2006). Several biomonitoring toxicological studies suggest that exposure to DINP is toxic and have various reproductive, developmental, carcinogenic, immunotoxic, neurotoxic and genotoxic effects in humans (CPSC, 2001). However, considerable amount of new data has emerged on the risk assessment of exposure to DINP on aquatic ecosystems, but with a very little knowledge on its aquatic toxicity. One of our previous studies concerning the concentration-dependent toxic effect of DINP (at 50, 100, 150, 200, 250 and 300 ppm/ L concentrations) when exposed to freshwater fish, *Oreochromis mossambicus* though not caused any lethal effect as mortality has been found to alter the normal behaviour as well as caused histopathological changes in gill and liver tissues (Revathy and Chitra, 2015). As continuation of previous findings, 300 ppm/ L concentration, which showed significant adverse effects, was chosen in the present study and are exposed to fish at different durations (24, 48, 72 and 96 h) in order to evaluate histopathological modifications in tissues of muscle and ovary in addition to liver and gill tissues. Consequently, the present study effectively provides valid information regarding the time-dependent toxic effects of DINP on histoarchitecture of various tissues in fish.

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MATERIALS AND METHODS

Maintenance of animal

Healthy adult freshwater fish, *Oreochromis mossambicus* (3.5 ± 0.75 g and length 5.5 ± 1.5 cm) were collected from a fish farm, Safa Aquarium, Kozhikode, Kerala. It was brought to the laboratory in plastic bags with sufficient air. The fishes were then transferred to glass aquarium tank of 40 L capacity, and were acclimatized to the laboratory conditions for 2 weeks with proper aeration and dechlorination. Fish were fed with commercial fish feed thrice a day during this period. Mortality

of fish was observed, dead fishes were removed and healthy fishes were selected for experiments after acclimatization. The physico-chemical features of the tap water were estimated before the start of experiment where water temperature ranged from 28 ± 2°C, oxygen saturation of water ranged between 70 and 100 %, pH is maintained at 7.6 which were monitored using standardized protocol (APHA, 1998).

Preparation of chemical

Diisononyl phthalate (DINP; CAS No. 28553120) of 99% purity was obtained from Sigma Aldrich chemical Co., USA. DINP (300 ppm) dissolved in propylene glycol (16 µl of 1 M) was used in the present study.

Experimental design

Experiments were carried out with 10 animals per group. Animals were grouped into two groups and each group with various subgroups as follows:

Group I: Control groups
Group a: Positive control group (with propylene glycol as solvent)
Group b: Negative control group (without solvent)
Group II: Treatment groups (DINP)
Group i: 300 ppm/ L for 24 h
Group ii: 300 ppm/ L for 48 h
Group iii: 300 ppm/ L for 72 h
Group iv: 300 ppm/ L for 96 h

Histopathology

After the end of every treatment, fishes from both control and treated groups were sacrificed and gill, liver, muscle and ovary were removed. Tissues were then fixed in buffered formalin, dehydrated in ascending alcohol series and cleared in xylene. Tissues were embedded in molten paraffin wax and sections of 5-6 µm thickness were made with a rotary microtome. Preparations were stained with eosin- hematoxylin and mounted in DPx and the stained sections were observed under trinocular research microscope and photographed.

RESULTS AND DISCUSSION

Diisononyl phthalate (DINP) is a high molecular weight phthalate due to its long backbone carbon chain. Recent biomonitoring data confirm that DINP and its primary and secondary metabolites were found in urine, breast milk, saliva and serum of human (Latini *et al.,* 2009). Therefore, the exposure to DINP could cause potential risks to the exposed animals, particularly for human health. Most of the studies have focused on the effects of DINP in human and there is limited data available on its effect on aquatic organisms. DINP has widespread use in most of the consumer products and therefore, it is likely discharged and released into the aquatic ecosystem. The solubility of DINP tested in our laboratory is 300 ppm/ L using propylene glycol as solvent. The selected concentration of DINP for 96 h did not caused treatment related alterations in the body weights of fish.

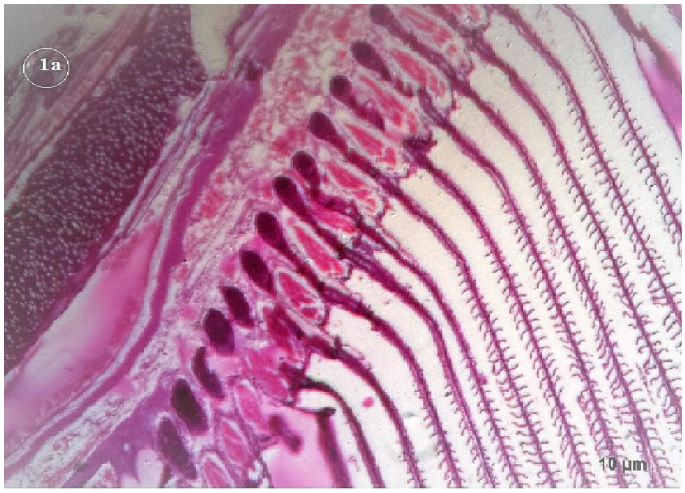

Fig. 1a. Photomicrograph showing normal architecture of gill of *Oreochromis mossambicus*

Fig. 1b. Photomicrograph showing normal architecture of gill of *Oreochromis mossambicus* exposed to propylene glycol

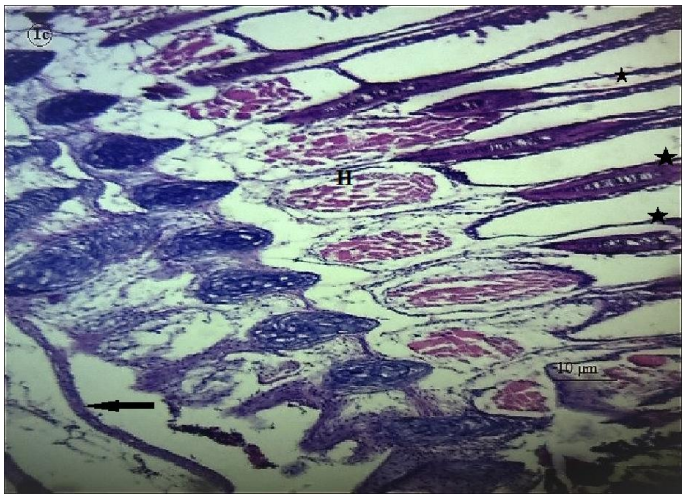

Fig. 1c. Photomicrograph showing upliftment of gill epithelium (arrow), hyperplasia in gill arches (H) and absence of secondary lamellae (asterisks) in gill of *Oreochromis mossambicus* after exposure to DINP at 300 ppm for 24 h

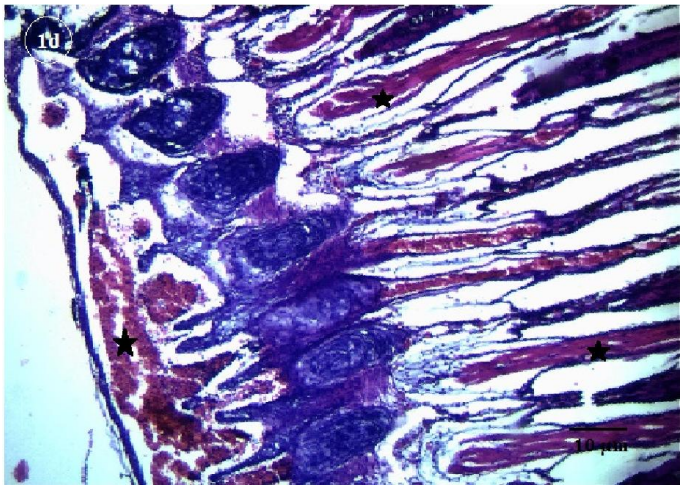

Fig. 1d. Photomicrograph showing aneurysm in gill epithelium and lamellae (asterisks) in gill of *Oreochromis mossambicus* after exposure to DINP at 300 ppm for 48 h

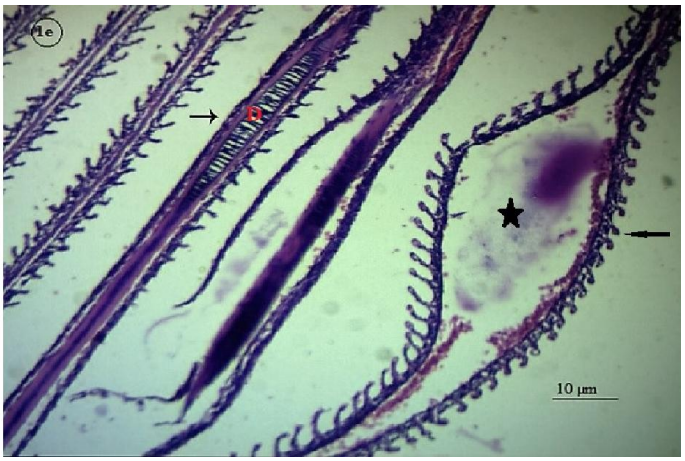

Fig. 1e. Photomicrograph showing absence of secondary lamellae (right arrow), curling of secondary lamellae (left arrow), dialation of blood vessel (D) and swollen deterioration in primary lamellae (asterisk) in gill of *Oreochromis mossambicus* after exposure to DINP at 300 ppm for 72 h

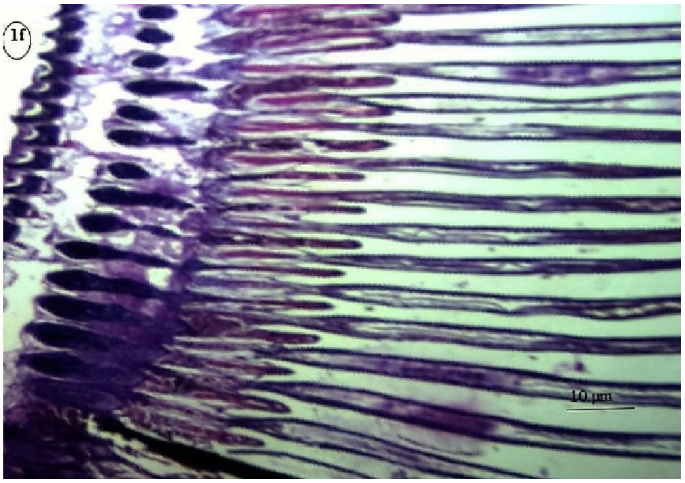

Fig. 1f. Photomicrograph showing complete absence of secondary lamellae in gill of *Oreochromis mossambicus* after exposure to DINP at 300 ppm for 96 h

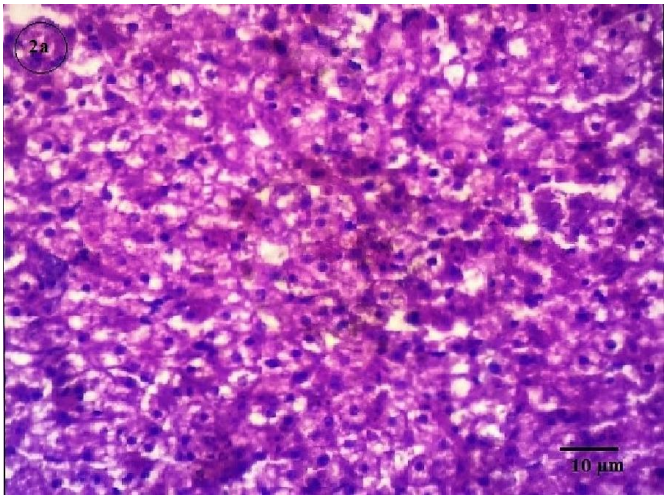

Fig. 2a. Photomicrograph showing normal architecture of liver tissue in *Oreochromis mossambicus*

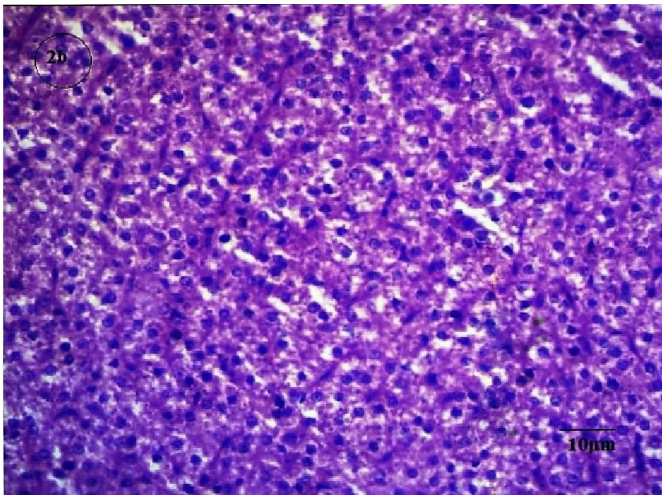

Fig. 2b. Photomicrograph showing normal architecture of liver tissue in *Oreochromis mossambicus* exposed to propylene glycol

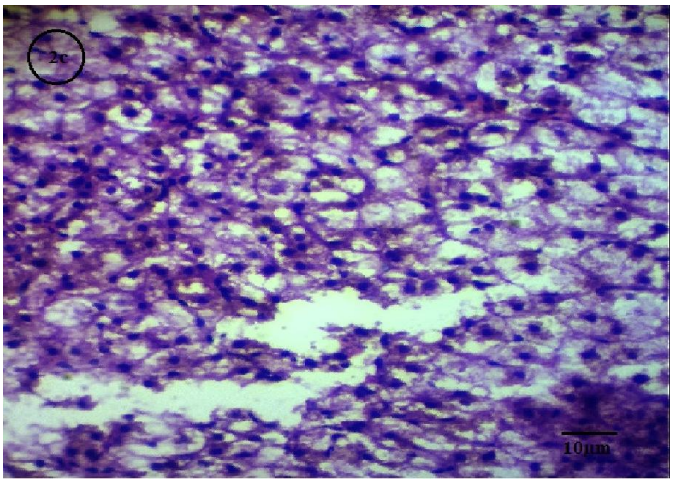

Fig. 2c. Photomicrograph showing degenerated cytoplasm in hepatocytes of *Oreochromis mossambicus* after exposure to DINP at 300 ppm for 24 h

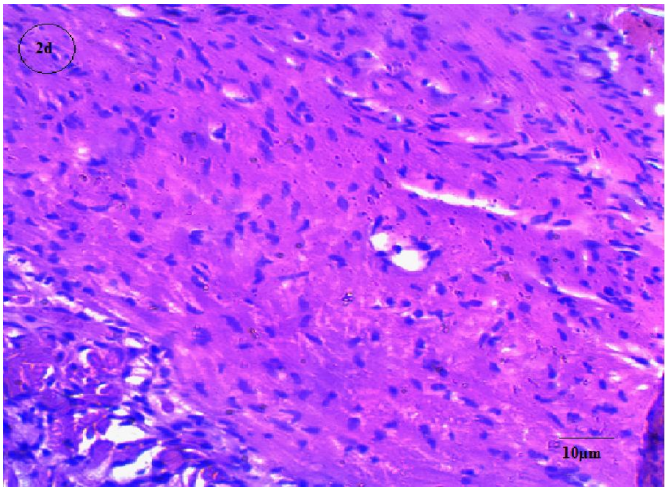

Fig. 2d. Photomicrograph showing spindle shaped nucleus in hepatocytes of *Oreochromis mossambicus* after exposure to DINP at 300 ppm for 48 h

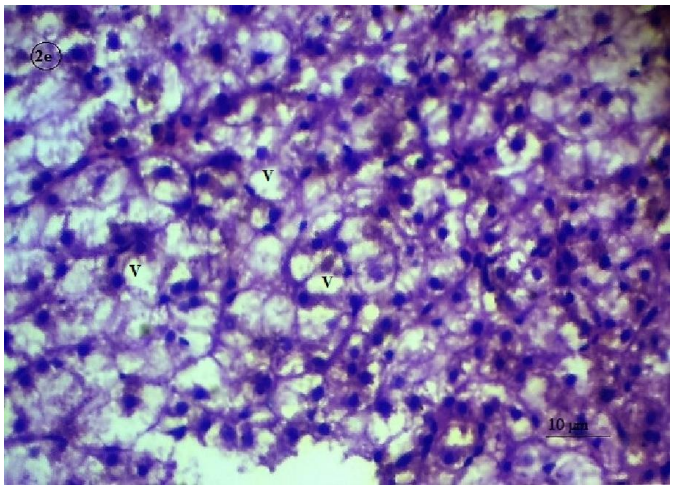

Fig. 2e. Photomicrograph showing cytoplasmic vacuolization (V) in hepatocytes of *Oreochromis mossambicus* after exposure to DINP at 300 ppm for 72 h

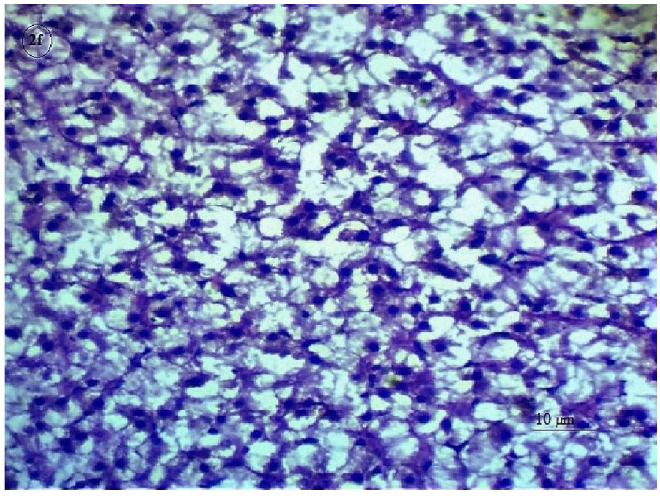

Fig. 2f. Photomicrograph showing necrosis in hepatocytes of *Oreochromis mossambicus* after exposure to DINP at 300 ppm for 96 h

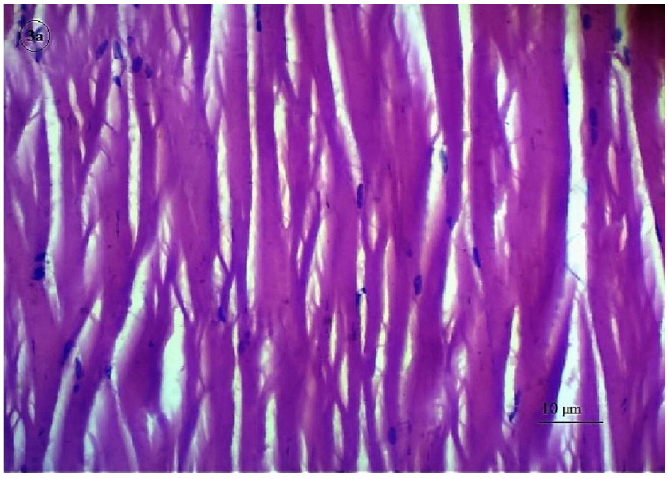

Fig. 3a. Photomicrograph showing normal architecture of control muscle tissue with muscle fibre and spindle nucleus

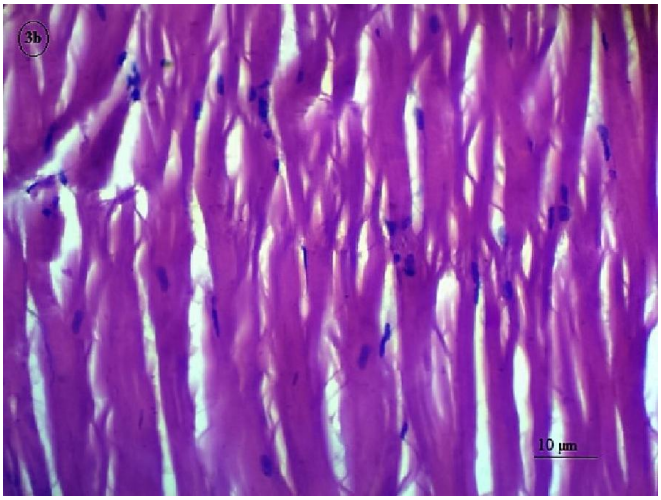

Fig. 3b. Photomicrograph showing normal architecture of muscle of *Oreochromis mossambicus* exposed to propylene glycol

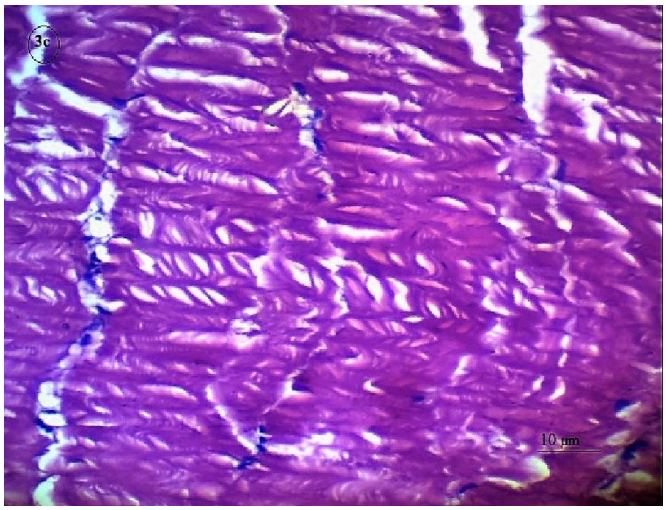

Fig. 3c. Photomicrograph showing degenerated muscle fibres after exposure to DINP at 300 ppm for 24 h in *Oreochromis mossambicus*

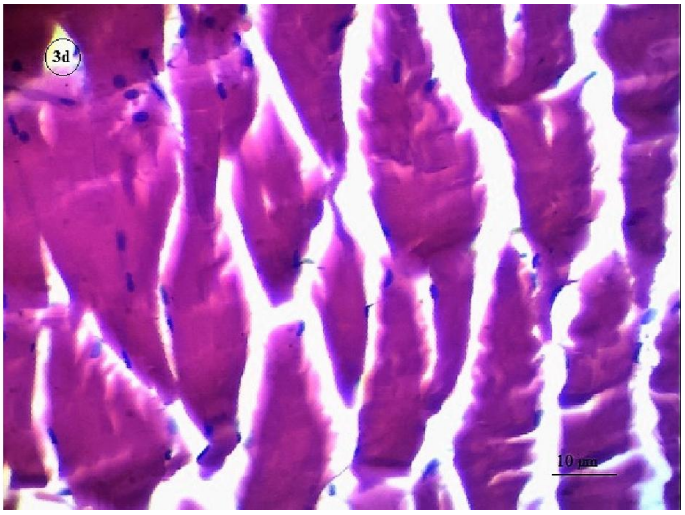

Fig. 3d. Photomicrograph showing larger space between the muscle fibres with irregular nucleus after exposure to DINP at 300 ppm for 48 h in *Oreochromis mossambicus*

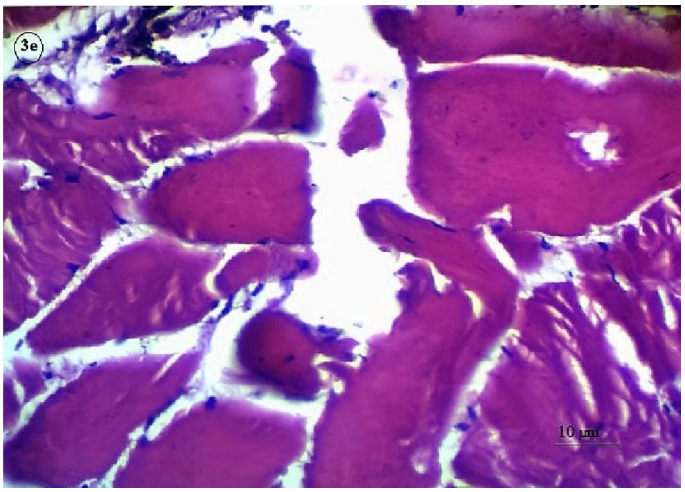

Fig. 3e. Photomicrograph showing shortened and thickened muscle bundles in *Oreochromis mossambicus* after exposure to DINP at 300 ppm for 72 h

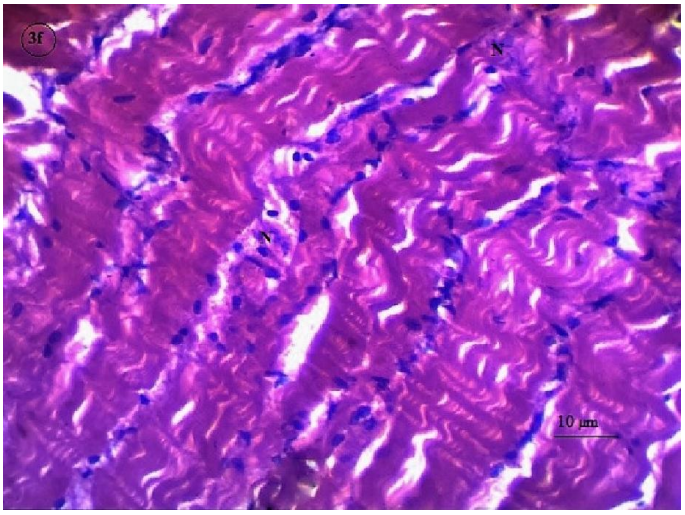

Fig. 3f. Photomicrograph showing disorganized and necrotic muscle bundles with irregular nucleus (N) in *Oreochromis mossambicus* after exposure to DINP at 300 ppm for 96 h

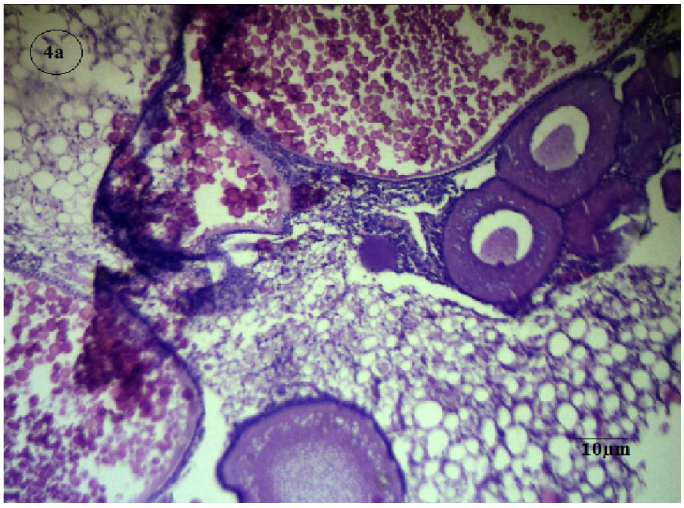

Fig. 4a. Photomicrograph showing normal architecture of ovarian follicle with prominent germ cells and vitellogenic oocytes of different stages in *Oreochromis mossambicus*

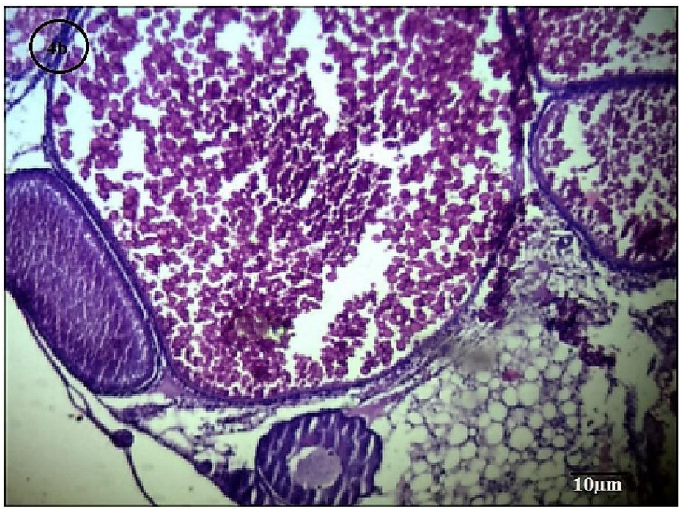

Fig. 4b. Photomicrograph showing normal architecture of ovarian follicle in *Oreochromis mossambicus* exposed to propylene glycol

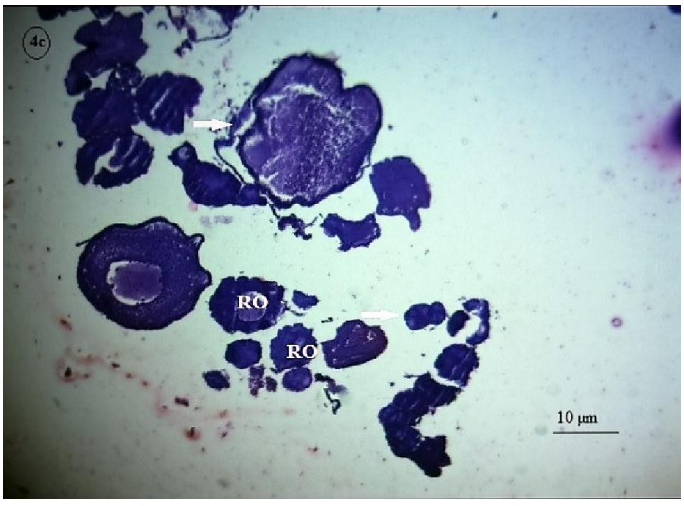

Fig. 4c. Photomicrograph showing highly reduced (RO) and loosely packed oocytes (arrows) due to loss of inter-follicular connective tissues after exposure to DINP at 300 ppm for 24 h in *Oreochromis mossambicus*

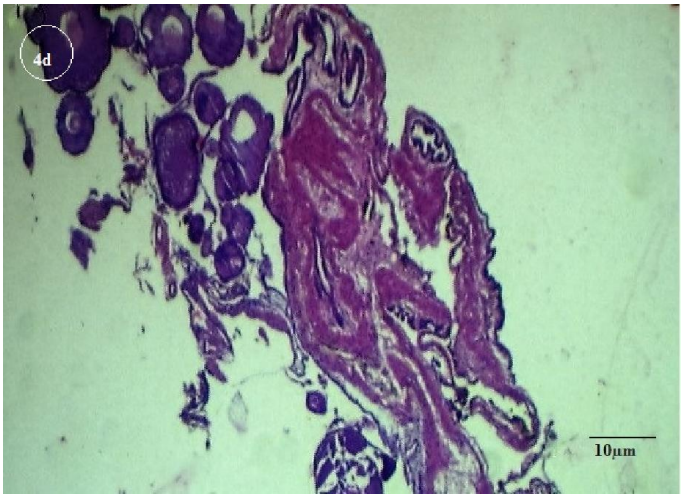

Fig. 4d. Photomicrograph showing degenerated oocytes after exposure to DINP at 300 ppm for 48 h in *Oreochromis mossambicus*

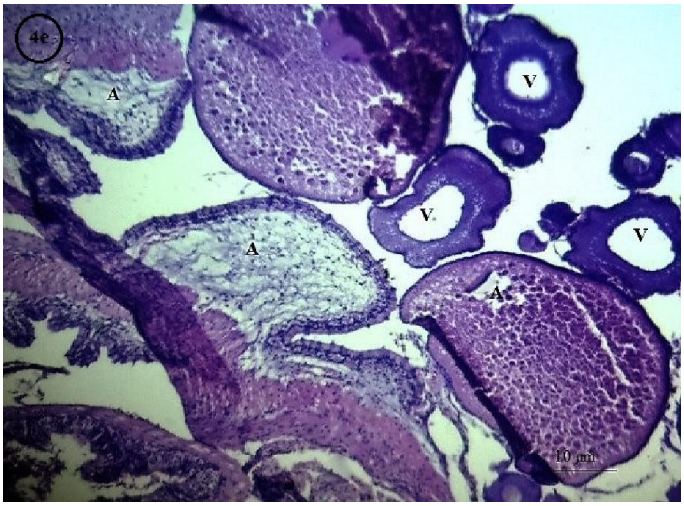

Fig. 4e. Photomicrograph showing atresia (A) and vacuolization (V) of oocytes in *Oreochromis mossambicus* after exposure to DINP at 300 ppm for 72 h

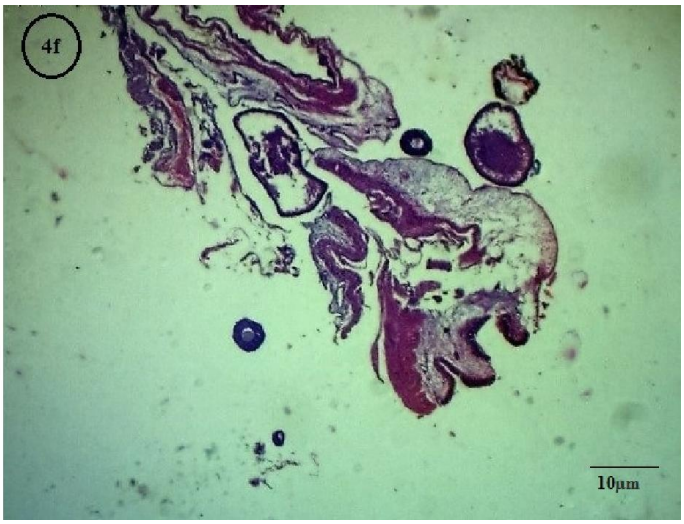

Fig. 4f. Photomicrograph showing reduction in number of ovarian follicles and distorted follicles in *Oreochromis mossambicus* after exposure to DINP at 300 ppm for 96 h

Similarly positive control fishes, treated with propylene glycol alone, did not caused any change in the body weights or organ weights for 96 h when compared to the negative control group (Revathy and Chitra, 2015). Acute toxicity is a prompt area of toxicological research for evaluating the impact of toxic chemicals on fishery resources. Acute toxicity test usually provides median lethal concentration of the test chemical in aquatic organisms during a specific period of time. However, DINP within the solubility limit of 300 ppm did not caused mortality in fish, *Oreochromis mossambicus*. As a result, LC_{50}-96 h was not found in the test chemical for 96 h. In the present study, histopathological observations were carried out at 300 ppm up to 96 h at different time interval in the tissues such as gill, liver, muscle and ovary.

The investigation of histopathology of various organs is a cost effective tool to determine the health of fish population, which reflects the health of entire aquatic ecosystem. Gill is the most sensitive organ, which reacts first to exposed toxic chemicals and where respiration, osmoregulation and excretion take place (Perry and Laurent, 1993). In the present study, control gill tissues (negative and positive controls) showed normal architecture having gill arches on either side of buccal cavity. Each gill arch is composed of numerous gill filaments with primary and secondary lamellae, inside which are chloride cells, erythrocytes, mucous cells etc. (Figs. 1a and 1b). DINP-treated fish for 24 h showed upliftment of gill epithelium, hyperplasia and loss of secondary lamellae (Fig 1c). At 48 h of DINP exposure showed epithelial and lamellar aneurysm (Fig 1d) and in 72 h of DINP exposure was observed with curling of secondary lamellae, absence of secondary lamellae, dialation of blood vessels with swollen deterioration in primary lamellae (Fig 1e). DINP treatment for 96 h showed complete loss of secondary lamellae (Fig 1f). The present examination showed that all structural modifications due to DINP exposure are in time-dependent manner. These histopathological changes are found similar when *Oreochromis mossambicus* was exposed to sublethal concentration of nonylphenol (Chitra and Mohan, 2014).

Liver is considered as the most important organ linked with detoxification, metabolism and excretion of toxic substances in the body (Van Dyk *et al.,* 2007). Its function, position and blood supply makes one of the reliable organs used to assess the impact of toxic chemicals and as representative of biological endpoints of contaminant exposure (Stentiford *et al.,* 2003). In the present study, control hepatocytes (negative and positive controls) showed normal architecture possessing parenchymatous polygonal hepatocytes with homogenous pink columnar cytoplasm and blue spherical nucleus (Figs. 2a and 2b). In 24 h treatment group, hepatocytes were observed with degenerated cytoplasm (Fig 2c). DINP when exposed for 48 h showed spindle shaped nucleus (Fig 2d) and in 72 h of DINP treatment made cytoplasmic vacuolization in hepatocytes (Fig 2e). After 96 h of DINP hepatocytes had undergone complete necrosis (Fig 2f). The degree of histopathological lesions was seemed to be related to the increasing exposure period of DINP treatment. The present histopathological alterations reveal that DINP exposures distress the normal configuration of liver tissue. Similar

observations have been reported on chlorpyrifos exposure to cichlid fish, *Etroplus maculatus* (Raibeemol and Chitra, 2015).

Muscle tissue is composed of elongated muscle fibres held together by connective tissues. Control muscle tissue (negative and positive controls) showed normal muscle fibre with spindle nucleus (Figs. 3a and 3b). On exposure to DINP at 300 ppm for 24 h showed flabby and degenerated muscle fibres (Fig 3c). After 48 h of DINP exposure, showed larger space between the muscle fibres with irregular nucleus (Fig 3d). DINP treatment for 72 h was observed with shortened and thickened muscle bundles (Fig 3e). Muscle bundles that are disorganized and necrotic with irregular nucleus were noted at the end of 96 h of DINP exposure (Fig 3f). In the present study histopathology of muscle tissue portrait the progressive damage in the structure of muscle with increase in exposure period. Similar observations have been observed when *Oreochromis* was exposed to chromium (Abbas and Ali, 2007). The present result documents the pathologic changes in muscle tissue is due to DINP exposure. Teleost oocytes are surrounded by outer thecal layers and inner granulose cells and during the growth of oocytes the follicle cells multiply and form a continuous layer called granulose cell layer (Selmann and Wallace, 1989). Normal histology of ovary reveals that the follicles are at different stages of growth. In the control groups (negative and positive controls), ovarian follicle showed follicle wall with prominent germ cells and vitellogenic oocytes of different stages (Figs. 4a and 4b). Fishes when exposed to DINP for 24 h showed highly reduced oocytes and are loosely packed due to loss of inter-follicular connective tissues (Fig 4c). DINP treatment for 48 h was observed with degenerated oocytes (Fig 4d). In 72 h DINP exposed group, atresia and vacuolization of oocytes were observed (Fig 4e). After 96 h of DINP treatment number of ovarian follicles was highly reduced and the existing follicle was found distorted (Fig 4f). The severity of lesions were found to be time-dependent and it was well known fact that follicular atresia are of vital importance for fish breeding and this degenerative process that occurs in ovary are likely to affect the fertility rate of fish (Guraya, 1994). Therefore, the present study clearly illustrates that DINP influence reproductive potential of fish.

Conclusion

Results of the present study hopefully contribute to the adverse effect of DINP at acute exposure to fish within the solubility limit of 300 ppm. In addition, it is well understood that if DINP is happened to expose chronically could irreversibly damage the normal architecture of vital tissues and also alter the fertility rate of fish as observed by histopathologic atresia in oocytes.

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