Research Square

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Evaluation of Toxicity and Oxidative Stress Following Chronic Exposure to Lipid-Lowering Drug Atorvastatin Using Nile Tilapia

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Abstract

Statins have attracted considerable attention in health care because of their pleiotropic effects. At the same time, concerns have been raised about the effects of prolonged statin use. This study aimed to understand the effects of Atorvastatin (ATV) in Nile tilapia (*Oreochromis niloticus*). The fish were exposed to 0.02, 0.05, 0.1, 0.2, and 0.5 mg/kg of ATV for 45 days. Overall growth, morphological alterations, and mortality were assessed at 45 days. Behavioural changes were also observed in treated groups compared with controls at the 15th, 30th, and 45th days of the experiment. Statin-induced toxicity was evaluated through histopathological analysis, and oxidative stress was assessed through superoxide dismutase (SOD) and catalase (CAT) activities. The findings of this study revealed dose-dependent mortality and alterations in morphological and behavioural patterns in fish exposed to high ATV concentrations. Dose-dependent, noticeable changes in tissue architecture were detected in the livers, intestines, and eyes of all treated fish. Lipid peroxidation increased only at 0.2 and 0.5 mg/kg of ATV, suggesting oxidative damage at these concentrations. The response of Nile tilapia to ATV-induced oxidative stress was assessed by examining SOD and CAT activities. No statistically significant changes were observed at 0.02 and 0.05 mg/kg, but increased activity of both enzymes was detected at concentrations of 0.1, 0.2, and 0.5 mg/kg of ATV. Together, our findings suggest that consuming ATV is safe at lower concentrations, even with long-term use, whereas higher concentrations may pose a serious threat that needs further investigation to address.

1. Introduction

Statins are well-recognised lipid-lowering agent that targets 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) to reduce cholesterol levels. The statins such as fluvastatin, pravastatin, simvastatin, lovastatin, atorvastatin, pitavastatin, and rosuvastatin are widely used in health care system [1]. Despite common mechanisms of action, statins differ in chemical structure and biochemical properties, which significantly impact the efficacy and safety of statin use [2]. While some previous findings highlighted the efficacy of statins in the reduction of inflammation, oxidative stress, thrombosis, and cardiovascular diseases, other findings raised concerns related to the adverse effects of statins, such as statin-associated muscle symptoms (SAMS), diabetes mellitus, cognitive decline, and haemorrhagic stroke [3]. Therefore, the use of statins has become a topic of debate among scientists and warrants further investigation for better application.

ATV lowers plasma low-density lipoprotein (LDL) by targeting HMG-CoA reductase, and it is completely absorbed after oral administration [4]. Pharmacokinetic studies suggest that 12% of ATV reaches systemic circulation, of which 95% is eliminated via faeces and 5% via urine [5]. The significant impact of ATV on cardiovascular diseases not only arises from its lipid-lowering effect but also from its pleiotropic effects. ATV also possesses immunomodulatory and anti-proliferative effects, which make it potentially useful in atherosclerotic plaque [6, 7]. Although the pleiotropic effects of ATV highlight its benefits, they can be overlooked. Previous studies also suggest that ATV use is associated with several adverse

effects on the gastrointestinal tract and liver function. However, the toxicity induced by chronic exposure to ATV remains unclear.

Nile tilapia, a freshwater fish, is widely used for toxicity evaluation because of its remarkable adaptability to diverse environmental conditions [8]. Importantly, the tilapia genome has been completely sequenced, providing a strong basis for research in genetics and molecular biology [9]. Furthermore, Nile tilapia is vulnerable to many common aquaculture diseases, making it a suitable model for studies on animal health, vaccine development, microbial infection, and the toxicity of pharmaceutical compounds. Environmental exposure to ATV may affect the gastrointestinal tract or liver function [10].

Although statins have received considerable attention in the field of health care system, the concern raised against the side effects of statins has converted them into a debatable topic. Therefore, the study has been designed to understand the effects of ATV used for a prolonged period on Nile tilapia as a model organism.

2. Materials and Methods

2.1. Collection of fish

The Nile tilapia fingerlings were collected from the National Freshwater Fish Brood Bank, Uttara, Odisha and transported to the laboratory with the help of an aeration container. The fish were acclimatised in 10 aquariums of 25-litre capacity, supplied with proper aeration and commercial feed twice a day. Nile tilapia with a weight of about 4.36 ± 0.33g and a 6.0 ± 0.5cm length were chosen for the experiment.

2.2. ATV treatment for fish

The fish received 0.02, 0.05, 0.1, 0.2, and 0.5 mg/kg body weight concentration of ATV through intraperitoneal injections for 45 days continuously. Each group included 20 fish, and one group remained as a control group. The ATV concentration used for fish was derived from a clinically relevant dose, ensuring realistic therapeutic exposure.

2.3. Morphological and mortality assessment.

Alterations in physical appearance, such as pigmentation, gill colours, caudal structure, and body shape, were documented and photographed using a mobile camera (50 megapixels). The dead fish were identified based on the absence of response to external stimuli and opercular movement. The mortality rate of Nile tilapia was calculated after 45 days of ATV treatment,

Mortality rate = number of fish dead / number of fish stocked X 100

2.4. Behavioural analysis

The behavioural pattern was strictly assessed through visual observation on the 15th, 30th, and 45th days of the experiment. Behaviour recorded on the first day (beginning of the experiment) was

considered the normal activity, and the behavioural responses of ATV-treated fish were compared with this baseline to detect behavioural deviations [8].

The five fish were transformed from the experimental tank to an aquarium with the same concentrations of statin doses to evaluate behavioural changes. The bottom, middle, and upper zone was labelled as 0–5 cm, 6–10 cm, and 11–15 cm, respectively. Gasping for air was observed when the fish remained at the top, using the opening and closing of the mouth and the number of opercular movements per minute. The schooling behaviour of fish was assessed by observing whether the fish swam randomly or exhibited a swimming pattern in the same direction. The appetite behaviour of fish was observed by providing commercially available food particles. The time gap between the first bite and the complete consumption of all food particles was recorded, and the longer the time gap, was considered a loss of appetite [8].

2.5 Histopathological analysis

Liver, intestine and eye tissue samples were taken from both control and ATV-treated fish and preserved in 10% formalin at room temperature. The collected tissues were prepared for paraffin embedding, and a microtome was used to generate 5-micron sections for histopathological analysis and staining. Slides were deparaffinized with a heat block for 1 min at 35°C and hydrated with deionised water. Then slides were exposed to haematoxylin for three minutes and eosin for two minutes. Excess staining was removed by gentle washing in running water. Then the slides were dehydrated with successive alcohol grading, cleared with xylene, and mounted using permanent mounting media. The Leica DM500 light microscope was used to observe histopathological changes, and images were captured at 10x and 40x magnification of the microscope.

2.6 Determination of oxidative stress markers

Measurement of Lipid peroxidation (LPX): The production of malondialdehyde was the key to measuring the level of LPX. The protocol provided by Buege and Aust was followed to measure the level of LPX.

Superoxide Dismutase Assay **(SOD Assay)**

The superoxide scavenging capacity of samples was measured using a spectrophotometer. Riboflavin was added exogenously, followed by the photoreduction to generate superoxide radicals in the sample. The SOD assay, followed by Paital et al, for this study was adopted for this study.

Catalase Assay **(CAT Assay)**

Catalase activity was detected in samples using a spectrophotometer by tracing the drop in absorbance at 240 nm. The methods described by Aebi et al were followed to perform the catalase assay.

2.7. Statistical analysis

The significant difference in data was calculated by one-way ANOVA using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, USA). The results were presented as mean ± SEM and were assessed as statistically significant using the p-value (ns: non-significant, *Significant at $P < 0.5$, **Significant at $P < 0.01$, and ***Significant at $P < 0.001$).

2. Results

3.1 Atorvastatin (ATV) reduced survival and induced morphological abnormalities in Nile tilapia.

To assess the effect of ATV on Nile tilapia, the fish were regularly exposed to increasing doses of ATV (0.02, 0.05, 0.1, 0.2, and 0.5 mg/kg body weight), and their mortality rates were monitored over 45 days after the initial exposure (Fig. 1A). The results showed that fish survivability remained unaffected at 0.02 and 0.05mg/kg ATV treatment, whereas a notable reduction in survivability was observed at 0.1, 0.2, and 0.5 mg/kg (Fig. 1B). Distinct morphological changes, such as haemorrhage, caudal erosion, discolouration, and mucus secretion, were observed in fish exposed to ATV (Fig. 1C). Our results suggest that atorvastatin treatment at lower concentrations does not significantly affect survival, whereas higher concentrations have a significant impact on both morphology and survivability.

3.2 ATV-induced behavioural changes in Nile tilapia.

The abnormal behaviours observed in Nile tilapia exposed to different doses of atorvastatin over time are shown in Table 1. The fish exposed to 0.02, 0.05, 0.1, and 0.2 mg/kg exhibited normal behaviour, but 0.5 mg/kg AT induced behavioural changes in fish. Notable behavioural changes at this dose included sluggish movement, unpredictable swimming, prolonged stay near the tank bottom, and increased surface gasping. The altered behaviour at 0.5 mg/kg ATV treatment persisted throughout the experiment. Overall, the results suggest that only at the highest ATV dose did Nile tilapia exhibit behavioural abnormalities.

Table 1

Behavioural changes in fish exposed to ATV at 15th, 30th & 45th day of treatment. N: Normal, S: Sluggish movement, LA: Loss of appetite, W: Weak, P: Present, B: Bottom DSB for disturbed school conduct. EL for Erratic Locomotion, SM for Slow-Moving Activity, SB for Steady at the Bottom, and GS for Gasping of Air. Restricted Appetite (RA).

Treatment	Time Points of Experiment		
	15 days	30 days	45 days
Control	N	N	N
0.02 mg/kg	N	N	N
0.05 mg/kg	N	N	N
0.1 mg/kg	N	N	N
0.2 mg/kg	N	N	N
0.5 mg/kg	GS, EL, SM, RA	GS, EL, DSB, SB, SM, RA	GS, EL, DSB, SB, SM, RA

3.3 ATV-induced histological alterations in Nile tilapia

Histological analysis of fish liver showed normal liver structure in the control group and in fish treated with 0.02 and 0.05 mg/kg of ATV (Fig. 2A-C). Treatment with 0.1 mg/kg ATV led to degeneration of pancreatic islands and hepatic cells (Fig. 2D). Treatment with 0.2 mg/kg and 0.5 mg/kg ATV resulted in hepatocyte degeneration and hepatopancreatic congestion (Fig. 2E **and F)**. Overall, histological analysis indicates that statin treatment at lower doses does not alter liver architecture even over extended periods. However, higher concentrations may be damaging to liver structure.

HE staining of the intestine revealed no alteration in the 0.02 mg/kg-treated fish compared to the control intestine (Fig. 3A-B**).** Disruption of longitudinal muscularis, rupture of villi, and fusion of villi were observed at 0.05 mg/kg treatment (Fig. 3C**).** The fish treated with 0.1mg/kg and 0.2 mg/kg showed abnormalities, including disruption of the longitudinal muscularis, dispersion of villi, shortening of villi, and truncated villi (Fig. 3D **and E)**. Histological analysis of the intestine exposed to the highest concentration of ATV (0.5mg/kg) induced significant damage in intestinal tissue architecture (Fig. 3F).

To check the effect of statins on sensory organs, a histopathological analysis of fish eyes was performed for both untreated and treated fish. Our results revealed that ATV treatment had a minimal effect on the structure of the eye tissue. Fish treated with 0.02, 0.05 & 0.1 mg/kg of ATV exhibited a similar eye layer compared to the standard eye structure (Fig. 4A-D). Higher ATV administration concentrations (0.2 mg/kg and 0.5 mg/kg) caused layer fusion, layer gap, damage to the pigmented epithelium, damage to the photoreceptor layer, decreased inner plexiform layer, layer loss, and nerve fibre impairment (Fig. 4E-F).

3.4 ATV induced oxidative stress only at higher concentrations.

Lipid peroxidation level was determined by the formation of TBAR. The result showed a constant lipid peroxidation level in both liver and intestine at 0.02, 0.05 & 0.1 mg/kg of ATV **(**Fig. 5A **and B)**. The significant rise in lipid peroxidation level was observed in higher concentrations of ATV (0.2 & 0.5 mg/ml. Anti-oxidant response was verified through SOD & CAT activity. In the groups treated with 0.02 mg/kg and 0.05 mg/kg of ATV, the SOD activity remained unchanged, with no significant differences compared to the control group. However, a significant increase in SOD activity was detected in both the liver and intestine at 0.1 mg/kg, 0.2 mg/kg, and 0.5 mg/kg of ATV **(**Fig. 5C **and D)**. In the liver, the catalase activity remained unaffected in the treatment group with 0.02 mg/kg and 0.05mg/kg ATV and elevated at 0.1 mg/kg, 0.2 mg/kg, and 0.5 mg/kg ATV **(**Fig. 5E **and F)**. Though a significant rise in catalase activity was observed at ATV 0.5 mg/kg when compared to the control, decreased catalase activity was noticed when compared to ATV 0.2 mg/kg. A similar trend was also observed in the intestine. Together, our data suggest that a lower dose of ATV administered over a prolonged period is safe, as it is not associated with oxidative stress.

3. Discussion

Statins are frequently used to treat cardiovascular diseases (CVD), but the repurposing of statins against other diseases, such as cancer and pulmonary inflammation have been tremendously increased in health care system [11–13]. Patients with diabetes and high blood pressure are more likely to develop heart disease, and statins are a valuable therapeutic option for them. However, many people who take these medications may experience specific adverse effects, such as nervous system problems, muscle soreness and weakness, and liver damage, which could lead to difficulties and exacerbate the side effects [14]. Though statins have a significant impact on cardiovascular diseases, the adverse effects of prolonged consumption of statins cannot be ignored [15, 16]. Therefore, the study attempted to understand the effect of chronic exposure to atorvastatin in Nile tilapia over 45 days.

Our findings suggest a clear dose-dependent response to ATV, as the survivability of fish was significantly affected by increasing doses of ATV. The survival of fish exposed to lower concentrations of ATV suggests metabolic adaptation and efficient detoxification mechanisms. Previous studies have reported the least mortality and non-significant morphological abnormalities in zebrafish embryos exposed to a low concentration of simvastatin [17, 18]. The increased mortality at higher ATV concentrations indicates impairment of vital physiological functions. ATV interferes with cholesterol biosynthesis pathways by targeting HMCo-Reductase, which is not only important for lipid metabolism but also for membrane structure, steroid hormone synthesis, and cell signalling [19]. Therefore, at elevated ATV concentrations, disruption of these processes may play a crucial role in the impairment of different physiological functions, leading to death of the organism. The observed morphological such as haemorrhage and caudal erosion suggest tissue damage, which may be the result of compromised

vascular integrity and oxidative stress [20]. Skin discolouration and mucous secretion at higher ATV concentrations reflect damage to epithelial tissue and mucosal barriers [21]. Skin discolouration in fish is also attributed to the defensive mechanisms of fish against various stressors and chemicals [22].

Behavioural responses are frequently used in toxicological studies because they are specific to toxicants and are guided by a specific pattern of molecular mechanisms [23]. In the present study, fish exposed to lower and moderate concentrations of ATV exhibited normal behaviour throughout the experiment, suggesting that these concentrations are not associated with neurological alterations or changes in routine activity [24].Similar to our findings, intraperitoneal injection of fluoxetine in cichlid fish reduced food intake, respiratory stress, body weight, and glycogen content [25].

Histopathological analysis is a reliable approach in toxicological studies, as it provides direct and visual evidence of tissue damage caused by toxicants. In the present study, normal hepatic architecture in fish exposed to lower concentrations of ATV suggests that the consumption of lower doses of ATV regularly does not affect the liver. Hepatic tissue damage at higher ATV concentrations indicates cellular dysfunction, metabolic stress, inflammation, and impaired circulation. Histopathological findings of this study are consistent with previous in vitro studies, reflecting the cytotoxicity of ATV and simvastatin in fish hepatocytes at only high concentration [26]. The tissue damage at high ATV concentration may be the result of an alteration in lipid metabolism and failure in oxidative stress response [17]. Similar to our findings, hepatocyte degeneration and inflammation were noticed in the liver of mice because of ATV-mediated oxidative stress and inflammation [27]. In the human physiological system, atorvastatin and simvastatin elevate the liver enzymes, but are very rarely associated with severe liver damage[28]. Preclinical studies have also reported the positive link between liver damage and dose escalation of statins, with a few cases of mortality [29]. The response of the fish system to the statins cannot be extrapolated to mammalian physiology, but the findings of the study, along with previous findings, support the ATV-mediated hepatotoxicity at a high concentration for a prolonged period.

In this study, histopathological analysis revealed the deterioration of intestinal tissue architecture in a dose-dependent manner. Distinct histopathological alterations induced by moderate doses indicate early tissue damage and impairment of the physiological detoxification mechanism. Similar to our findings, shortening of villi, epithelial damage, and damage in the tunica were observed in *Clarias gariepinus* exposed to atrazine for 28 days [30]. ATV treatment caused developmental abnormalities and metabolic alterations in the *Mugilogobius chulae* embryo through inhibition of autophagy and apoptosis. [31] .The findings of this study, such as disruption of the longitudinal muscularis, dispersion of villi, shortening of villi, and truncated villi at 0.1 mg/kg and 0.2 mg/kg of ATV, corroborate previous findings that suggest fish exposed to pharmaceutical compounds, pesticides, xenobiotics, and heavy metals cause intestinal damage [32–34]. Similar disruptive architecture was also observed in fish exposed to chronic and high-dose exposure to pharmaceutical drugs and environmental pollutants, leading to epithelial damage, inflammation, and compromised gut function [35].

The eye's sensitivity to chronic exposure to pharmaceutical drugs is a concern, as toxins accumulate and cross the blood-retina barrier. In this study, the histopathological alteration was detected only at higher concentrations. These histopathological deviations may compromise visual function and neural transmission. A previous toxicological study has also documented intact retinal layers in fish exposed to low doses of pharmaceutical toxicants [35]. Interestingly, retinal degeneration has been reported in fish exposed to high concentrations of statins due to impaired lipid metabolism and oxidative stress [36, 37]. Therefore, our findings align with previous studies that highlight the sensitivity of fish sensory organs to elevated levels of pharmaceutical toxicants.

Oxidative stress is a key indicator of stress caused by pollutants, temperature, hypoxia, and pharmaceutical toxicants. The findings reflect a dose-dependent TBAR formation in the liver and intestine in Nile tilapia. The significant increase in TBAR formation at 0.2 and 0.5 mg/kg of ATV highlights the impaired antioxidant defence system and damage in lipid membrane. It also suggests that beyond a threshold concentration, ATV disrupts redox homeostasis, leading to an increase in TBAR formation. Similar dose-dependent TBAR level elevation has been reported in fish exposed to paracetamol [38]. Our findings differ from a previous randomised clinical study, where ATV decreased plasma TBARS levels in hyperlipidaemia after 12 weeks of treatment [39]. Chronic exposure to ATV significantly decreased the serum TBRS level in a patient diagnosed with coronary artery disease [40]. However, the increase in lipid peroxidation at high concentrations in this study suggests that the effect of chronic exposure depends on tissue, physiological condition, dose, and time of exposure.

SOD and CAT are two major components of the antioxidant system that counteract the overproduction of ROS in cells. The joint venture of SOD and CAT neutralises superoxide radicals during oxidative stress. Both SOD and CAT activity remained unchanged at lower ATV concentrations and decreased at higher concentrations. The reduction of this enzyme activity at high concentrations may be the result of enzyme saturation, dysfunction of the anti-oxidative system to encounter increased ROS [41]. The effect of statins on these enzymes has been reported as a biphasic response. One of the systematic reviews reported increased SOD activity following ATV treatment, whereas the same study also reported no change in SOD activity [42]. Similar to our findings, ATV was reported to increase ROS levels and decrease the activities of SOD and CAT [27]. It suggests that ATV may induce oxidative injury and impair the antioxidant capacity at high pharmacological doses. The elevated SOD and CAT activity at high ATV in this study can be interpreted as an adaptive upregulation of anti-oxidative response. Such an adaptive response was previously documented [43].

Conclusion

In this study, the toxic effects of ATV were evaluated through histological and biochemical analyses, and our findings suggest that long-term use of ATV at lower concentrations is safe, whereas higher concentrations may pose a serious threat. Therefore, our study recommends careful management and monitoring of ATV in clinical practice. Furthermore, ATV exposure for more than 45 days, followed by the detection of immunomodulation, will provide a definitive conclusion regarding ATV-associated toxicity.

Future research is highly essential to identify the genetic changes through RNA sequencing, which will provide information regarding the involvement of genes in statin toxicity.

Declarations

Competing interests-

The authors declare no competing interests.

Ethical approval-

This study does not contain human samples. The guidelines of the “Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)”, Government of India, and an Institutional Animal Ethics Committee (IAEC) vide registration no. 433/GO/Re/S/2001 /CCSEA, were strictly followed to conduct the animal experiment in this study.

Consent to participate-

This study does not include any human subjects

Consent to publish-

This study does not include data of any individual except the authors.

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Author Contribution

Conception, design, and development of methodology, data analysis, and interpretation: IB, FK, PN, SS, RM, and DM. Fish collection, toxicity experiment: IB, FK, PN, AM, JS, SS, and DM. Biochemical analysis, histological analysis: IB, FK, JS, SS, RM, and DM. Manuscript Drafting: IB, FK, PN, AM, RM, and DM. Study supervision: DM.

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Data Availability

All data generated or analysed during this study are included in this published article.

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Figures

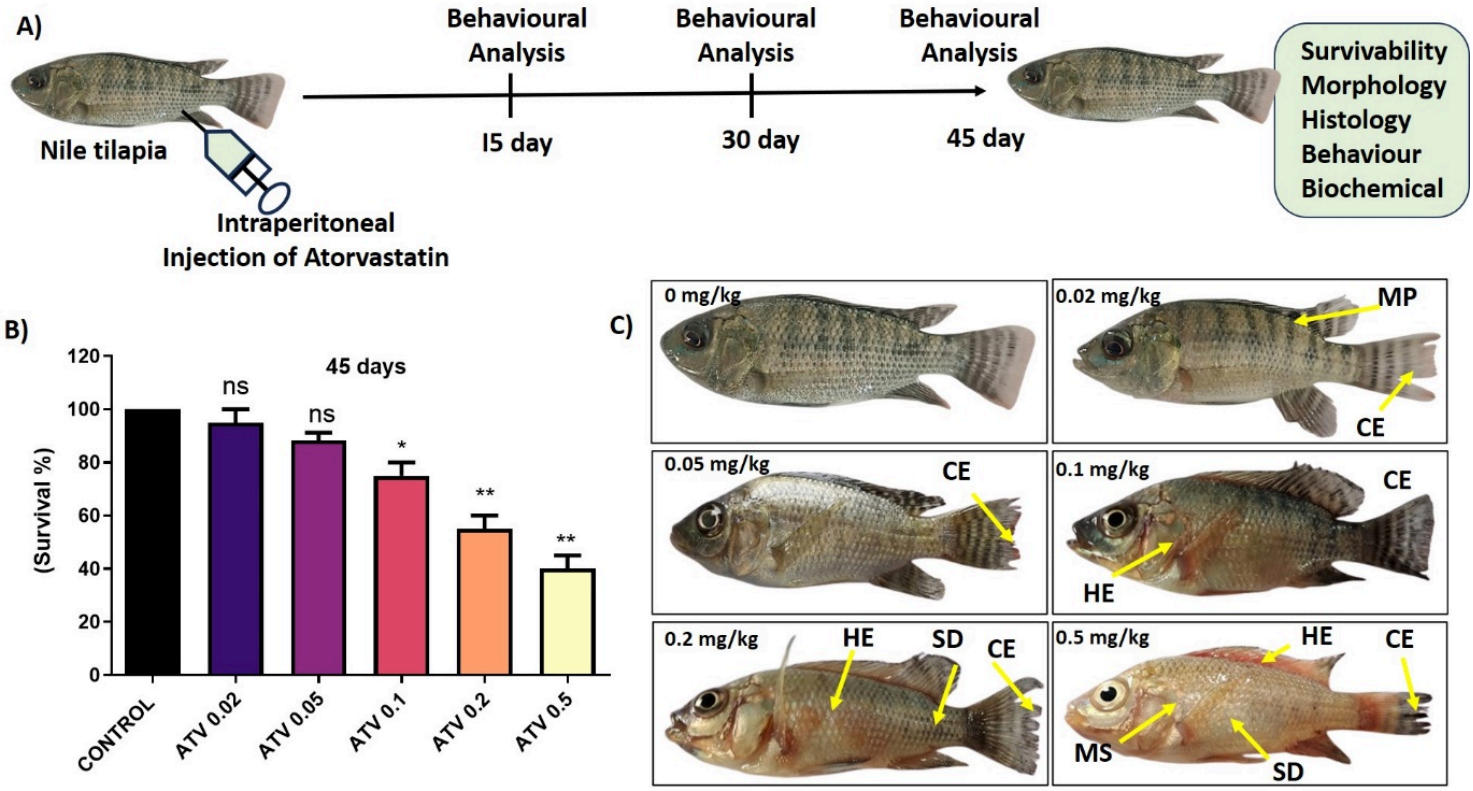


Figure 1

ATV-induced toxicity in Nile tilapia and an illustration of the investigation." A) A summary of the experimental configuration, B) The percentage of fish that survive after being exposed to an ATV, and C) Morphological alteration exhibited by fish treated with different concentrations of ATV. Haemorrhage (HE), skin discolouration (SD), caudal erosion (CE), melanin pigmentation (MP), and mucus secretion (MS). *Significant at P<0.5, **Significant at P<0.01, ***Significant at P<0.001, and ns: non-significant.

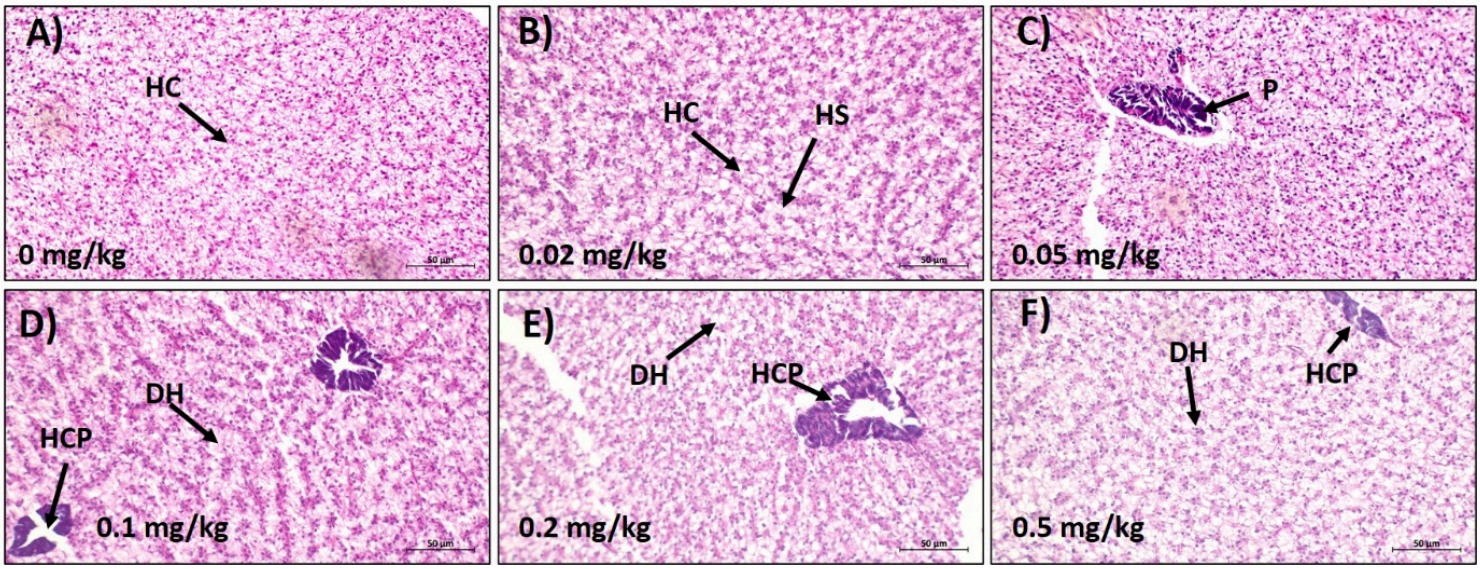


Figure 2

Microscopic images of liver sections from control and ATV-treated Nile Tilapia. A: Untreated/Control fish, having the normal liver architecture. B-F: Treatment with ATV showing alternation in liver architecture. HC: Hepatocytic cells, P: Hepatopancreatic tissue. HS: Hepatopancreatic sinusoids, DH: Degenerated hepatocyte. HCP: Hepatopancreatic congestion. Scale bar: 50µM.

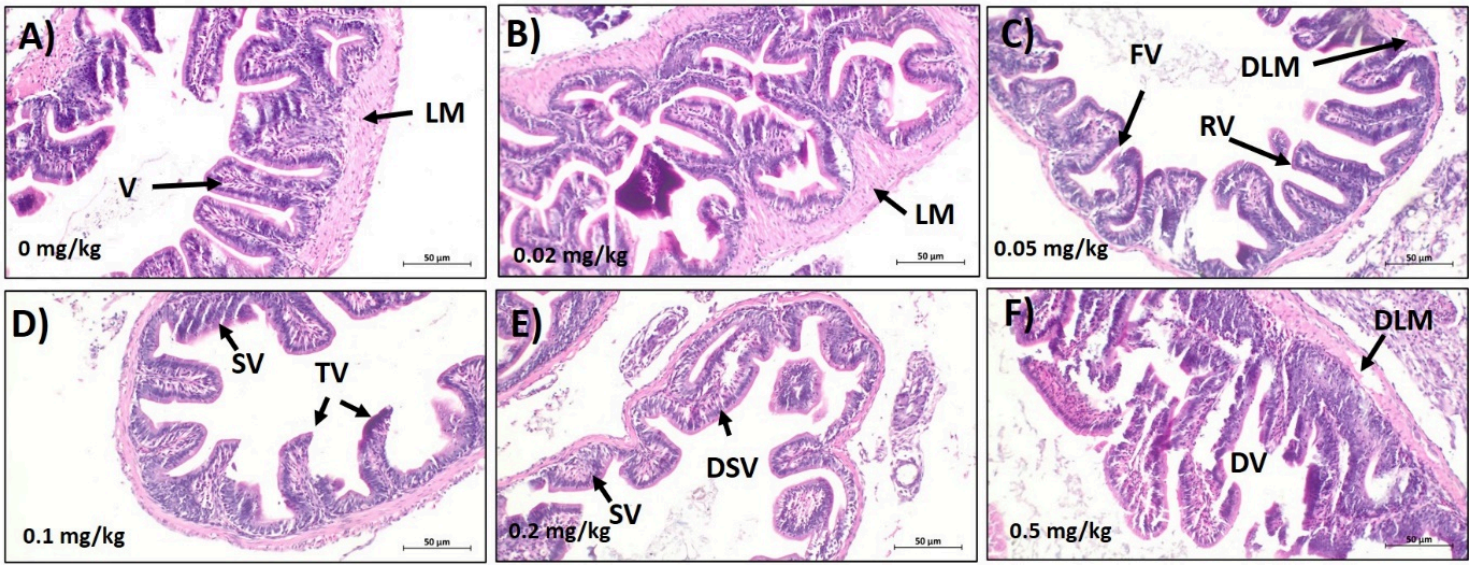


Figure 3

Representative microscopic images of the intestine in the control and ATV-treated groups. A: Tissue section of the intestine of a control fish. **B-F:** Histological sections of the intestines of fish treated with ATV. Longitudinal muscularis (LM), conventional villi (V), Disruption of longitudinal muscularis (DLM), ruptured villi (RV), fusion of villi (FV), shortened villi (SV), truncated villi (TV), dispersed villi (DV). Scale bar: 50 µm.

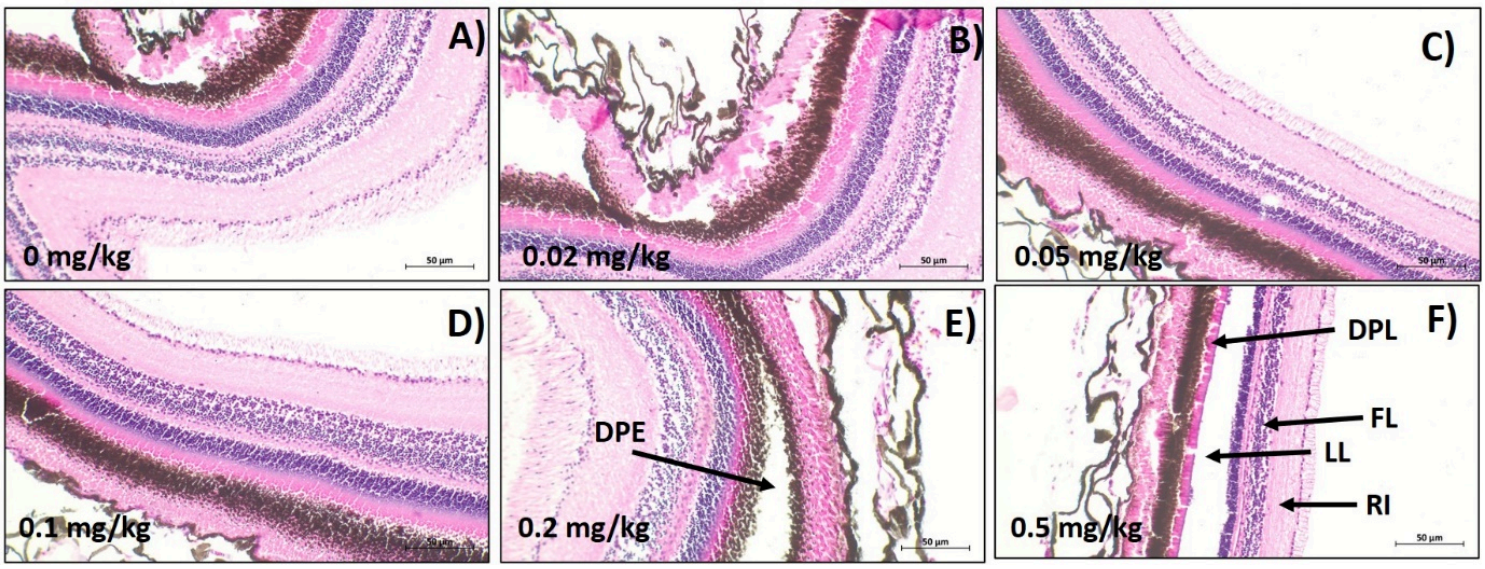


Figure 4

Representative microscopic images of the eye in control and ATV-treated groups. A: Figure represents different layers of the control eye, B: Eye layers of the untreated fish. A-B: Different layers of the eye and levels. C-G: Fish administered ATV have distinct layers of the eye. FL: Layer fusion, LL: Layer loss, RI: Diminished inner layer of the plexiform DPL: Damaged photoreceptor layer; DPE: Damaged coloured epithelium. Scale bar: 50μM is the

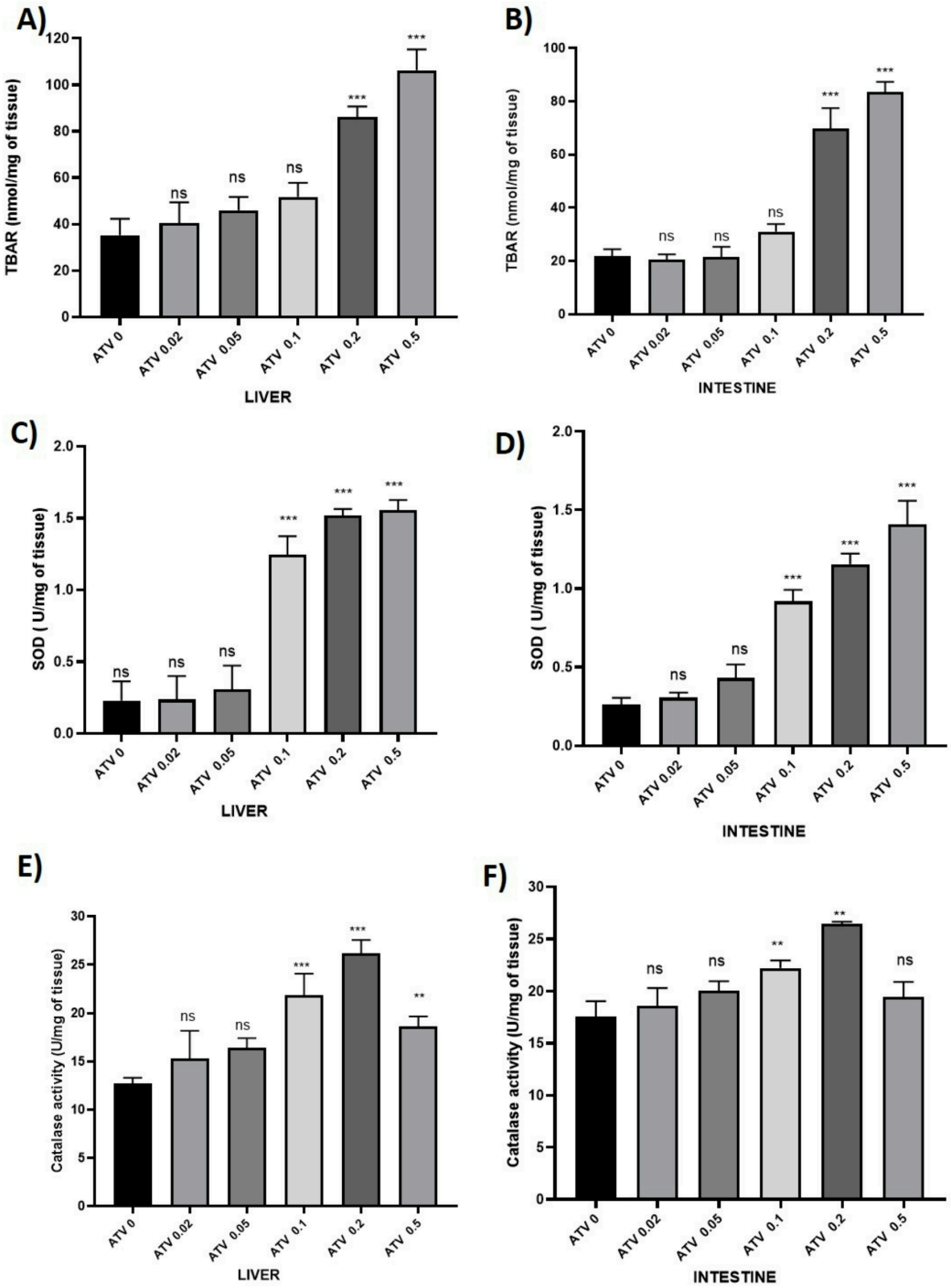


Figure 5

Atorvastatin-mediated oxidative stress in Nile tilapia. Lipid peroxidation in liver (A) and intestine (B). SOD activity in the liver (C) and intestine (D). Catalase activity in the liver (E) and the intestine (F). *Significant at P<0.5, **Significant at P<0.01, ***Significant at P<0.001, and ns: non-significant.