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Effect of Sulphuric Acid-Induced Acidic Environment on the Antioxidant Activity and Mitigating Role of Vitamins (C and E) in *Oreochromis niloticus*

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Abstract

ANTHROPOGENIC activities acidify water bodies by altering pH levels, primarily through industrial and agricultural chemical discharge and indirectly via acid rain. Fish are one of the most crucial and sensitive bioindicators in toxicological studies. This study evaluated the effects of sulphuric acid-induced acidic environment on the antioxidant activity and the mitigating role of vitamins (C+E) in *Oreochromis niloticus.* The T_0 (control) received a basal diet at optimal pH without vitamin supplements. The *O. niloticus* fingerings (T_1, T_2, and T_3) were exposed to varying pH levels (pH 6.5, 6, and 5.5) for 21 days. Antioxidant parameter results showed a significant decrease in total antioxidant capacity (TAC), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the acid-exposed groups compared to T_0 (control group). The role of combined and isolated vitamins (C+E, E, and C) in mitigating sulphuric acid-induced toxicity (T_1, T_2, and T_3) was examined for a period of next 21 days. Antioxidant parameter results showed a significant increase in TAC, CAT, SOD and GPx in the vitamin-supplemented groups compared to T_0. This study highlighted that a sulphuric acid-induced acidic environment (low pH) significantly disrupted the antioxidant systems of *O. niloticus.* These findings confirm that vitamins (C+E) neutralize oxidative stress due to acid stress effectively. Vitamin C showed more significant effects on antioxidant parameters than vitamin E.

Keywords: antioxidant status, ROS, mitigate, oxidative stress, catalase, superoxide dismutase, glutathione peroxidase.

Introduction

Aquaculture is providing a considerable stock of seafood to the world as a rapidly growing industry among food production industries, while relieving stress on wild fish supplies [1,2]. The water quality is declining and aquatic life is losing diversity because of pollution from waste, land changes, air pollution and contaminated runoff [3]. Water pH is thought to be the primary variable influencing water quality [4]. Freshwater acidification is a global issue affecting Europe, the USA and Asia. Fish and other aquatic life thrive in near-neutral pH levels and even slight changes disrupt their biological functions [5]. *Oreochromis niloticus* is chosen as the experimental model due to its widespread availability and suitability for toxicity tests [6]. Freshwater acidification mainly results from acid rain due to SO_2 and NO_x emissions and climate change via CO_2 uptake and humic acid from decaying organic matter [7]. Aquatic species biochemical and physiological responses are influenced by pH fluctuations in their environment. A sharp shift in pH can result in unexpected oxidative reactions in the oxygen-dependent metabolic process, leading to oxidative stress [8]. ROS (Reactive oxygen species) production and the rise in metabolic requirements brought on by warmth and acidification

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typically result in peroxidation-induced damage and an increase in the activity of antioxidant enzymes [9]. The changes in pH increase ROS production, damaging fish DNA, proteins and lipids, which can lead to genetic disorders [10]. Antioxidant defenses in fish are a vital mechanism to prevent ROS-induced DNA damage caused by oxidative stress, as they scavenge reactive oxygen species (ROS) and free radicals [8]. Antioxidant enzymes work to eliminate ROS, which are produced by acidification of the oceans and environmental warming. However, fish can be harmed by excessive ROS production as it exceeds the capacity of their antioxidant defenses [11]. Cells are often protected against lipid peroxidation by the enhanced activity of antioxidant enzymes brought on by ROS generation [12,13]. Superoxide dismutase (SOD) transforms the extremely reactive radicals of superoxide (O_2^-) produced by mitochondria into hydrogen peroxide (H_2O_2). In order to stop O_2^- from building up in tissues and cells, catalase (CAT) transforms H_2O_2 that has been transformed by SOD into non-toxic water and oxygen [11]. Glutathione peroxidase (GPX) facilitates the breakdown of hydrogen peroxide (H_2O_2) into water (H_2O) or transforms organic peroxides into their corresponding stable alcohols [14].

Antioxidant capabilities are strongly exhibited by vitamins, phenolic compounds, and carotenoids, which are important bioactive chemicals [15]. These substances oxidize easily because they have a hydroxyl group at the C-6 (carbon-6) position. This group is important because it helps the body function normally by removing radicals. As a result, it plays a key role in protecting cell membranes from lipid damage [16]. Vitamin C, also known as L-ascorbic acid (AA) may help boost the immune system and limit tissue oxidative damage [17]. A naturally occurring antioxidant, vitamin C deactivates destructive free radicals that are oxidative, which are produced by normal cell processes and other stresses [18]. Since vitamin C gives free radicals an electron and subsequently two protons to generate chemically dehydroascorbic acid. It is commonly considered a vital primary protective component that decreases or restores free radicals. This is because vitamin C is a powerful antioxidant that inhibits the free radicals generation, which result from oxidative damage to lipids and lipoproteins across various cells and tissues [19]. Vitamin E refers to eight fat-soluble antioxidant forms, including tocotrienols and tocopherols. The tocopherol strongly enhances the antioxidant systems of fish [16]. Vitamin E functions as an antioxidant, preventing free radicals formed during regular metabolism or stressful events like stress, pollutants and infection from oxidizing macromolecules. These macromolecules including lipoproteins, unsaturated fatty acids and nucleic acids [20].

The tissues and cells can be protected against oxidative stress by applying exogenous substances having antioxidant capabilities, like vitamins C and E, which can scavenge free radical activity [21]. Thus, the present study aims to investigate the effects of varying acidic environments on the antioxidant activities of the fish *O. niloticus* and the mitigating role of combined and isolated vitamins (C and E).

Material and Methods

Experimental design and fish

Tilapia (*Oreochromis niloticus*) fingerlings were used in this study, taken from the Punjab fish hatchery on Satyana Road in Faisalabad. This study was conducted in zoology laboratory of University of Agriculture Faisalabad, Punjab, Pakistan. The fingerlings were acclimated in the laboratory environment for one week. Fingerlings were maintained in a well-aerated glass tank and fed commercial feed. Eighty fingerlings were distributed into four trial groups. T_0 was considered as control group, maintained on a basal diet at neutral pH without any supplementation. The pH was reduced to acidic levels (6.5, 6, 5.5) in treatment groups (T_1, T_2 and T_3) using a 10% H_2SO_4 solution for 21 days. The pH tends to revert to its original pH, so pH was measured three times daily using a Milwaukee MW150 MAX pH/ORP/temp meter and maintained with 10% H_2SO_4 solution as needed. This experiment was conducted at room temperature and water parameters were maintained at optimal levels (except pH). After 21 days, three fingerlings per trial group were dissected for analysis. Fingerlings were acclimatized for 24 hours without feed before dissection. The largest specimens were selected, gently netted to minimize stress and benzocaine was used to anesthesia fish. Their livers were collected for analysis of antioxidant activity. The three fish sample from each group is actually the replication of results in order to apply statistical analysis and to enhanced the reliability of results. Acidic exposure was then discontinued and fingerlings were supplemented with vitamins C and E for an additional 21 days and assessed the mitigating role. The T_1 was supplemented with a combined dose of 1260mg/kg of vitamins C and E. T_2 received 420mg/kg of vitamin E and T_3 840mg/kg of vitamin C alone. Post-supplementation, fingerlings from each group were dissected and evaluated the mitigating effects of the vitamins.

Antioxidant Activity

Antioxidant activity was measured by hepatic total antioxidant capacity (TAC) and by the hepatic antioxidant enzyme system (CAT, SOD and GPx). The collected liver tissues were stored in cold saline, and homogenates were prepared from each sample Different antioxidant enzymes including total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase

(GPx) [22]. The status of various antioxidant enzymes was analyzed from liver tissue samples using an enzyme-linked immunosorbent assay (ELISA, #MBS038818 for CAT, #MBS705758 for SOD and #MBS1601701 for GPx) and colorimetric assay kit (Catalog Number EEA022 for TAC) according to the manufacturer instructions.

Tissue Samples

The 0.02–1 g of fresh liver tissue was used and rinsed with PBS (0.01 M, pH 7.4) at 2–8°C to eliminate blood cells. The excess water was removed using filter paper and the weight was measured. The tissue was homogenized in PBS (0.01 M, pH 7.4) (2–8°C) at a ratio of 9 mL buffer per 1 g of tissue, then centrifuged the homogenate at 10,000 g for 10 minutes at 4°C. The supernatant was collected and kept on ice for analysis of TAC, CAT, SOD and GPx. Avoid multiple freeze-thaw cycles.

Statistical Analysis

The statistical analysis provided the data through mean ± standard error of mean. One-way ANOVA with repeated measures was used to compare values between the treatment group and the control group. ANOVA was followed by the comparison of means following Tukey's test using Minitab software version 17.

Results

Antioxidant responses to pH changes of the fingerlings Nile tilapia, *O. niloticus* are shown in Table 1. The hepatic total antioxidant capacity and antioxidant enzymes including CAT, SOD and GPx was significantly decreased with the decreased in pH from 6.5 to 5.5. The lowest mean values of TAC, CAT, SOD and GPx were observed in T_3 at pH 5.5, followed by T_2 at pH 6.0 and then T_1 at pH 6.5. The control group maintained the highest mean values of TAC, CAT, SOD and GPx. The ANOVA indicated statistically significant differences ($p < 0.05$) among the pH-treated groups (T_1, T_2 and T_3) and control for TAC, CAT, SOD, and GPx. The p-value measured for TAC, CAT, SOD and GPx was 0.0001 which indicated the highly significant results.

Statistical analysis shows that means that do not share a letter are significantly different ($p<0.05$). Data were analyzed using one-way ANOVA followed by Tukey test for comparisons. Superscript uppercase letters indicate significant differences between groups; data are shown as mean (± SEM).

The supplementation with combined and isolated vitamins (C and E) significantly enhanced the antioxidant response in O. niloticus in Table 3. The vitamin-supplementation restored hepatic total antioxidant capacity and antioxidant enzyme levels (CAT, SOD and GPx) to the normal range compared to acidic exposure. In TAC and GPx the combined vitamin C+E treatment (T1, post-acidic exposure at pH 6.5) showed significantly higher mean values compared to the individual vitamin C (T3, post-acidic exposure at pH 5.5), vitamin E (T2, post-acidic exposure at pH 6.0) and untreated control (T0) groups. In CAT the vitamin (C+E) treatment (T1) showed higher mean values compared to vitamin C treatment (T3), untreated T0 and vitamin E treatment (T2). While in SOD the vitamin (C+E) treatment (T1) showed higher mean values compared to vitamin E treatment (T2), untreated T0 and vitamin C treatment (T3). The ANOVA indicated statistically significant differences ($p < 0.05$) among the pH-treated groups (T1, T2 and T3) and control for TAC, CAT, SOD, and GPx. The p-value measured for TAC, CAT, SOD and GPx was 0.0001 which indicated the highly significant results.

Statistical analysis shows that means that do not share a letter are significantly different ($p<0.05$). Data were analyzed using one-way ANOVA, followed by a Tukey test for comparisons. Superscript uppercase letters indicate significant differences between groups; data are shown as mean (± SEM).

Discussion

Antioxidant system parameters serve as a reliable biological indicator of oxidative damage in studies evaluating responses to various toxicants and different species [23]. The production of reactive oxygen species is a crucial stress response that causes oxidative damage to macromolecules [24]. The activities of antioxidant enzymes, including CAT, SOD and GPx, help in the detection of oxidative stress. When aquatic organisms respond to stress, their levels fluctuate, making them useful indicators [25]. The antioxidant enzymes such as SOD, GPx and CAT mitigate the increased levels of ROS caused by oxidative stress [26]. In organisms, intermediate oxidative stress increases the activity of enzymes that are antioxidants, while chronic oxidative stress reduces their ability to function, potentially resulting in apoptosis [27,11]. These mechanisms suggest that fish well-being and survival may suffer from elevated levels of oxidative stress [13,12]. According to this study, groups exposed to acid (pH 5.5, 6.0, and 6.5) had lower levels of total antioxidant capacity and activity of antioxidant enzymes (CAT, SOD, and GPx) than the control group. The TAC, CAT, SOD, and GPx levels were significantly reduced in T3 at pH 5.5.

The significantly reduced TAC value in T3 at pH 5.5 may be the result of acid-related oxidative damage, as free radicals are increased as a result of elevated acidity that damages the cell membranes and enzymes like SOD and CAT. The study's findings supported those of [28], founded that Nile tilapia exposed to elevated concentrations of AgNPs (AgNPs-50 and AgNPs-100) had considerably lower TAC levels than controls.

The considerably low value of CAT at pH 5.5 in T3 may be the result of oxidative damage to liver cells or acid-induced structural alterations in proteins that inhibit production of antioxidants. The present study findings align with [29], who found that Astyanax altiparanae exposed to an acidic pH environment for 24 hours had reduced activity of CAT enzymes than the control.

The considerably decreased value of SOD in T3 at pH 5.5 may be the result of pH alterations that inhibit activity of SOD by damaging enzymes or reducing their synthesis. The findings are in agreement with [8] demonstrated that P. olivaceus may exhibit oxidative stress as a result of pH variations, which reduces activity of SOD by interrupting with the functioning of the enzyme. The results are contrary to those of [29], since SOD activity did not decrease following 96 hours of exposure to aluminum (Al), manganese (Mn), or an acidic pH.

The noticeably reduced value of GPx in T3 at pH 5.5 might be a result of acidic stress, which is caused by damage to cells or metabolic disturbance, that downregulates synthesis of GPx. The study results are similar to those of [29] indicated that Astyanax altiparanae showed a substantial decrease in activity of GPx following a 24-hour of Al+Mn exposure ($P < 0.01$).

Vitamins are essential organic substances for growth of animals including fish. The vitamins C and E are considered vital antioxidants. Vitamin C (VC) is a crucial antioxidant supplement utilized in fish meals and in food sector to mitigate oxidative stress [30]. According to [31], the main protective nutrient is vitamin C. Its antioxidant capabilities boost immunity, combat aging and prevent from infections by protecting cells from ROS. According to [25], vitamin E, as an antioxidant, protects cells from ROS-induced stress related damage. According to studies, it is a vital fat-soluble component that also strengthens immunity of fish. The groups supplemented with vitamins had higher levels of total antioxidant capacity and activities of antioxidant enzymes (CAT, SOD and GPx) than the control group and also post-acid exposed groups.

The significantly higher value of TAC was observed in T1 supplemented with a combination of vitamin C+E. The higher values of TAC might be due to capabilities of vitamins C and E together to neutralize ROS (reactive oxygen species) produced by acidic stress. The chronic exposure may be the reason for reduced value of T2 supplemented with vitamin E only, which could have resulted in serious harm. The results of this study are similar to the findings reported by [32], indicating that supplementing with VE and VC is an effective approach to mitigate oxidative stress in a variety of fish species. Vitamin E functions as an effective feed supplement that boosts growth and promotes early survival [33]. According to [34], dietary Cu-NPs and VC may raise levels of antioxidants and have a combined effect on Oncorhynchus mykiss's antioxidant mechanism.

The value of CAT was considerably higher in T1 supplemented with a combination of vitamin C and E. The reason behind this might be vitamins C and E work synergistically to lower dangerous ROS levels. This might have enhanced the production of CAT for breaking the waste product of metabolic processes, that is, hydrogen peroxide. The reasons for reduced value of T2 (vitamin E) could be chronic acid exposure that might have resulted in serious adverse effects. The study results also align with [35], enhanced activity of CAT in the OFO+C500+E400 group suggested that moderate doses of vitamins (C and E) effectively mitigate oxidative stress induced by OFO in rainbow trout. The enhanced levels of CAT, POD and SOD in Cyprinus carpio treated with vitamin C following exposure to FP and BPFN, showed vitamin C antioxidant abilities to scavenge free radicals effectively as reported by [36].

The considerably higher SOD values in T1 supplemented with the vitamin C+E combination. Vitamin C and E might be the reason of this, as they enhanced the activity of antioxidant enzymes like SOD, which eliminates superoxide radicals. The reason behind the reduced value of T3 supplemented with vitamin C only is the long-term acid exposure, which could have resulted in serious damage. The study results align with [37], indicating that dietary consumption of vitamin C raises SOD, CAT and GPx activity in yellow catfish (Pelteobagrus fulvidraco). The findings are in agreement with [38] revealed that oxidized diet containing 60 mg kg^{-1} of dietary vitamin E (VE) enhanced the activities of SOD and CAT in the liver of Lateolabrax japonicus.

The noticeably higher value of GPx was observed in T1 (vitamin C+E). This may be because combination of vitamins C and E boosts GPx production by lowering damage from free radicals and restoring each other antioxidant capabilities. According to [39] vitamin C is an antioxidant that helps protect animal cells from damage caused by harmful molecules (ROS) by breaking them down and neutralizing them. This study results are similar to those of [40] dietary supplementation with vitamin E slightly enhances the activity of GPx without working in combination with selenium. Vitamin C, nutritional supplementation to Ctenopharyngodon idella enhanced the expression of genes in the spleen and kidney, such as GPX1a and GPx4b as reported by [41].

Conclusion

The present study suggested that 21-day acid exposure severely compromised Nile tilapia (*O. niloticus*), causing oxidative stress through

antioxidant system disruption. These findings highlight the chronic toxicity of acidic environments in aquatic ecosystems. However, dietary vitamin supplementation with vitamin C (840 mg/kg), vitamin E (420 mg/kg) and their combination (1260 mg/kg) effectively mitigated these effects to some extent. The vitamins restored and mitigated oxidative damage, demonstrating their therapeutic potential against prolonged acid stress. Vitamin C showed more significant effects on antioxidant parameters than vitamin E. Furthermore, studies are needed on the molecular pathways through which vitamin C and E mitigate oxidative stress and immunosuppression. The mechanism behind vitamin C showing more significant effects on hematological and antioxidant parameters than vitamin E is also a topic of interest.

Conflict of Interest

No conflict of interest.

Authors Contribution

AY conceptualized and conducted the study and wrote the article. KB, M, and MK assisted with the trial, data collection, and analysis, and also helped conduct the experiment. AA and HB reviewed the article.

TABLE 1. Antioxidant parameters of Nile tilapia fingerlings exposed to different pH levels are represented as (Mean ± SEM).

Antioxidant Parameters	Treatments			
	T_0 (Control)	**T_1 (pH 6.5)**	**T_2 (pH 6.0)**	**T_3 (pH 5.5)**
TAC	1.55 ± 0.02^{A}	1.31 ± 0.006^{B}	1.06 ± 0.09^{C}	0.71 ± 0.01^{D}
CAT	148.3 ± 2.34^{A}	127.7 ± 1.45^{B}	95.57 ± 1.46^{C}	73.3 ± 1.20^{D}
SOD	63.7 ± 1.21^{A}	60 ± 0.57^{AB}	55.6 ± 0.89^{B}	48.6 ± 1.48^{C}
GPx	0.69 ± 0.009^{A}	0.54 ± 0.01^{B}	0.46 ± 0.02^{C}	0.33 ± 0.03^{D}

TABLE 2. Confidence intervals of antioxidant parameters.

	Confidence Intervals 95% CI			
Antioxidant parameters	**T_0 (Control)**	**T_1 (pH 6.5)**	**T_2 (pH 6.0)**	**T_3 (pH 5.5)**
TAC	(1.4479, 1.6587)	(1.20461, 1.41539)	(0.9613, 1.1721)	(0.6046, 0.8154)
CAT	(144.49, 152.18)	(123.82, 131.51)	(91.82, 99.51)	(69.49, 77.18)
SOD	(61.18, 66.16)	(57.509, 62.491)	(53.176, 58.157)	(46.18, 51.16)
GPx	(0.6653, 0.7213)	(0.5120, 0.5680)	(0.4354, 0.4913)	(0.3020, 0.3580)

TABLE 3. Antioxidant parameters of Nile tilapia fingerlings exposed to different pH levels are represented as (Mean ± SEM).

Antioxidant parameters	Treatments			
	T_0 (Control)	**T_1 (Vitamin C+E)**	**T_2 (Vitamin E)**	**T_3 (Vitamin C)**
TAC	1.67 ± 0.02^{C}	2.80 ± 0.058^{A}	1.15 ± 0.14^{D}	2.3 ± 0.056^{B}
CAT	156.7 ± 1.45^{C}	177 ± 0.58^{A}	143.3 ± 1.76^{D}	165 ± 1.73^{B}
SOD	68 ± 0.57^{C}	103 ± 1.15^{A}	86.6 ± 1.44^{B}	62.3 ± 0.89^{D}
GPx	0.71 ± 0.02^{D}	1.94 ± 0.03^{A}	0.94 ± 0.01^{C}	1.75 ± 0.04^{D}

Table 4. Confidence intervals of antioxidant parameters

	Confidence Intervals 95% CI			
Antioxidant parameters	**T_0 (Control)**	**T_1 (Vitamin C+E)**	**T_2 (Vitamin E)**	**T_3 (Vitamin C)**
TAC	(1.4905, 1.8562)	(2.6171, 2.9829)	(0.970, 1.336)	(2.1171, 2.4829)
CAT	(153.29, 160.04)	(173.627, 180.373)	(139.96, 146.71)	(161.63, 168.37)
SOD	(65.539, 70.461)	(100.54, 105.46)	(84.21, 89.13)	(59.872, 64.794)
GPx	(0.6647, 0.7553)	(1.8980, 1.9886)	(0.8947, 0.9853)	(1.7047, 1.7953)

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