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The Effect of Quercetin on Oxidatıve Stress in IPNV-infected Cells of Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract

In this study, the antioxidant effects and the free radical scavenging capacity of quercetin were investigated in IPNV-infected Rainbow Trout Gonad-2 (RTG-2) continuous cell culture. For this purpose, four different groups were formed (Control, IPNV, Quercetin, IPNV+Quercetin). Malondialdehyde (MDA), Total Antioxidant Status (TAS), Total Oxidant Status (TOS), Ceruloplasmin (Cp), and Glutathione Peroxidase (GPx) activities were measured at 0, 24, 48, 72, and 96 hours. According to the results of our study, there was a significant increase in TAS, TOS, and Cp results in the groups containing IPNV-infected cells. According to TAS and TOS measurements, there was a statistically significant increase both between groups and at different hours. When GPx results were analyzed, it was observed that there was no statistically significant change between the groups and between the measurement hours. When the MDA results were analyzed, it was observed that there was no change in the measurements made at different hours, while there were significant differences between the groups. It was observed that quercetin can minimize the damage caused by scavenging free radicals in the environment and thus provides an important development for fish farming and economy in cells infected with IPNV in the laboratory environment.

Introduction

Aquaculture is the fastest-growing animal food production industry. Between 2000 and 2019, global aquaculture production increased from 43 million tons to 120 million tons (FAO, 2022). It is an extraordinary growth, reflecting the rise in demand for seafood as a source of protein and the decline of wild fish stocks due to overfishing and environmental changes (Delgado et al., 2003). In Türkiye, approximately 70% of aquaculture farms are trout farms (TÜİK, 2023). The good climate and availability of a lot of freshwater resources make it highly conducive to trout farming. One of the first fish species reared in this country is the rainbow trout, still farmed the most today (Aydoğan et al., 2020; Korkmaz et al., 2008). However, with the increase in industrial growth and production, possible undesirable factors such as sudden temperature changes in culture conditions, poor water, and stress cause the emergence of infectious diseases such as Infectious Pancreatic Necrosis (IPN), which cause significant economic losses (Duran et al., 2022).

Infectious fish diseases such as IPN not only cause major economic losses in the aquaculture sector but also lead to restrictions in international trade. Infectious pancreatic necrosis (IPN) is an acute, contagious, systemic viral disease characterized by necrosis of the pancreas in different fish species, which is of great concern in aquaculture. The causative agent can frequently cause mortality of up to 80-90% of stocks, especially in juvenile rainbow trout (*Oncorhynchus mykiss*) and post-smolt Atlantic salmon (Salmo salar) production (Dopazo, 2020). Microscopically, lesions in salmonid fish occur primarily in the pancreas and

intestinal mucosa. In the pancreas, extensive necrosis of acinar and islet cells of Langerhans and intracytoplasmic inclusion bodies are observed (Erer, 2002; Roberts, 2001; Santi et al., 2004). These tissue damages caused by the virus in the body lead to the emergence of oxygen derived free radicals that have harmful effects on cell function (Mittal et al., 2014). These free radicals are physiological products of aerobic metabolism. However, their uncontrollable growth causes oxidation of biomolecules and disrupts their structure. The unpaired electrons in free radicals take electrons from the stable compound to become stable, stabilizing itself and making the compound whose electrons are removed into a free radical state (Kükürt et al., 2021). Oxidative stress is caused by an imbalance between oxidants and antioxidants (Akpınar et al., 2024). Cell membranes and organelles are damaged as a result of oxidative stress and impaired antioxidant defense mechanisms (Kükürt & Karapehlivan, 2022). The interaction initiated by free radicals continues until it is stopped by antioxidants. For this reason, the organism has developed an antioxidant defense system (ADS) to counteract the damage of free radicals. Antioxidants can suppress lipid peroxidation by inhibiting the peroxidation chain reaction or by capturing reactive oxygen species (Akkuş, 1995). The antioxidant defense mechanisms of fish include many of the factors that eliminate or limit the spread of agents that cause damage to host tissue, prevent infection from occurring, or enable the body to respond to infection (Blazer, 1992; McDowell, 2012). ADS can prevent cellular damage by controlling free radical circulation. In some cases, free radical production exceeds the capacity of the ADS and this is defined as oxidative stress (Atabek, 2011). There are a number of antioxidant compounds in the cell that capture oxidants and react with them to neutralize them (Davies, 1986; Kappus & Diplock, 1992; Quintanilha, 1981). Flavonoids, which show antioxidant properties, show free radical collecting properties. Flavonoid radicals formed by the separation of hydrogens are stabilized by forming chelate rings with trace metals in the environment (Giorgio, 2000). In addition, flavonoids have anti-inflammatory properties through various mechanisms such as inhibition of regulatory enzymes and transcription factors that play an important role in the control of mediators involved in inflammation (Maleki et al., 2019). Quercetin, one of the flavonoids found in green plants, is a very powerful antioxidant (Ergüzel, 2006). Therefore, it is a bioactive compound widely used in botanical medicine and traditional Chinese medicine (Xu et al., 2019). Quercetin is predicted to reduce oxidation by directly inactivating free radicals (Muderrisoglu et al., 2022). Knowing the specific pathways quercetin and other flavonoids act through may contribute to the development of targeted therapies in the management of diseases resulting from oxidative stress in aquaculture. Improved diet formulations that include them will thus improve fish health and resilience, hence providing a basis for gaining support for aquaculture operations and productivity.

In terms of the reliability of clinical trials, *in vitro* studies are a prerequisite for *in vivo* studies (De Munck et al., 2005). *In vivo*, studies are difficult to control and evaluate, expensive, and ethically controversial. The determination of the biological properties of the material is usually started with *in vitro* test methods, and then evaluations are continued with animal tests, which are more expensive and require a longer time (Schmalz & Arenholt-Bindslev, 2009). In this study, we aimed to determine the effect of quercetin on oxidative damage using cell culture (RTG-2) obtained from IPNV-infected rainbow trout cells *in vitro*.

Materials and Methods

Rainbow Trout Gonad-2 (RTG-2) continuous cell culture and IPNV SDF4 strains (GenBank: MH614932.1) in the stocks of Samsun Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Virology were used as materials in this study. Cell cultures were grown with 1% penicillin-streptomycin (10.000 U/mL) (Gibco, Ref. No: 15140-122), sodium pyruvate (Gibco, Ref. No: 11360-070), nonessential amino acid (Lonza, NEAA), 1% L-glutamine (Gibco), 1% sodium bicarbonate solution (Sigma) and 10% fetal calf serum (FBS, Sigma) in DMEM (Dulbecco's Modified Eagle Medium, Gibco, Ref. No: 41965-039). Cell Counting Kit-8, Sigma-Aldrich (Cat. No: 96992, USA) was used to determine the cytotoxic dose of quercetin. The viability of 50 µmol quercetin-treated cells was 52% after 24 hours and 50% after 48 hours, while the viability of 100 µmol quercetin-treated cells was 47% after 24 hours and 49% after 48 hours (Yıldırım & Gümüşova, 2020) (Figure 1). In our study, quercetin at a dose of 50 µmol/L was administered to the group of IPNV-infected cells for treatment.

In the study, control, IPNV, quercetin, IPNV+quercetin, IPNV+quercetin 4 different groups were formed for experimental purposes (Table 1.).

In this study, malondialdehyde was analyzed by the method reported by Yoshioka (Yoshioka et al., 1979). In the thiobarbituric acid (TBA) reaction, when the lipid content is heated at low pH and in the presence of TBA, a stable red-pink color with a minimum peak at 535 nm is produced. The red-pink color is due to the chromogen formed by the combination of two TBA molecules with the MDA molecule.

Ceruloplasmin in serum was measured spectrophotometrically by the modified Ravin method. Ceruloplasmin as an enzyme oxidizes phenylene diamine, a colorless substance, to a blue-violet colored product. This effect in serum was stopped by the addition of sodium azide at a certain time in the experiment and at the beginning of the experiment in the experimental blind and measured spectrophotometrically (Ravin & Harvard, 1956).

Total oxidant status (TOS) and total antioxidant status (TAS) were measured spectrophotometrically with the rel assay diagnostic kit as recommended by Erel

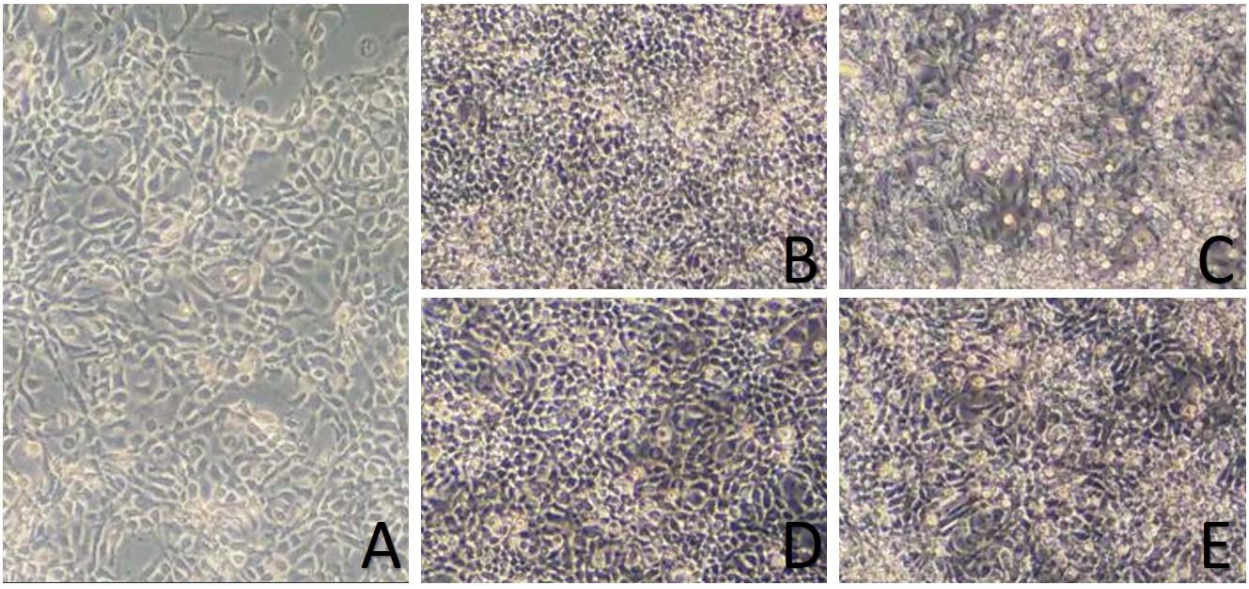

Figure 1. The viability of quercetin-treated cells (A. Control 24 hours, B. 50 µmol quercetin-treated cells for 24 hours, C. 50 µmol quercetin-treated cells for 48 hours, D. 100 µmol quercetin-treated cells for 24 hours, E. 100 µmol quercetin-treated cells for 48 hours)

Table 1. Experimental groups

Group 1	Control
Group 2	IPNV
Group 3	Quercetin (50µmol)
Group 4	IPNV+Quercetin (50µmol)

(2005) (Erel, 2005). In the assay, oxidants present in the sample oxidize the ferrous ion-chelating complex to ferric ion. The oxidation reaction is prolonged by enhancer molecules, which are abundant in the reaction medium. The ferric ion forms a colored complex with chromogen in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample.

Glutathione peroxidase (GPx) test was analyzed with an ELISA kit according to the procedures (Glutathione Peroxidase Assay Kit Item No. 703102). Oxidized glutathione (GSSG) released after the reduction of GPx and hydroperoxide is recycled as reduced by GR and NADPH. The resulting change in absorbance (ΔA340) per minute was calculated (Ursini et al., 1985).

Statistical Data Analysis Methods

SPSS 25.0 program was used for statistical analysis. Regarding the analysis of the data; descriptive statistics were determined with mean and standard deviation values. Kruskal Wallis test analysis was performed to examine whether the measurements in the study were different according to the characteristics of the study groups. Friedman test was performed to determine the difference between the measurements made at different times within the group. The pairwise method was applied to determine the different times and p-values less than 0.05 (P<0.05) were considered significant. The power level and effect size calculations calculated in the study were determined with G*Power (Version 3.1.7.).

Result

In the study, it was determined that group 1 MDA mµ/L measurements were not different at baseline, 24, 48, 72, and 96th hours (P=0.13). Group 2 MDA mµ/L measurements were not different at baseline, 24, 48, 72, and 96 hours (P=0.08). Group 3 MDA mµ/L measurements were found to differ according to the measurement hours. It was determined that the 48th hour measurements within the group were higher than the other measurement times (P=0.01). Group 4 MDA mµ/L measurements were not different at baseline, 24, 48, 72, and 96 hours (*P=0.13*). It was determined that the baseline, 24, 48, 72, and 96th measurements performed in the study did not differ according to the groups (P>0.05) (Figure 2).

In the study, it was determined that group 1 TAS mmol measurements were not different at baseline, 24, 48, 72, and 96 hours (P=0.11). Group 2 TAS mmol measurements were not different at baseline, 24, 48, 72, and 96 hours (P=0.42). Group 3 TAS mmol

measurements were not different at baseline, 24, 48, 72, and 96 hours (P=0.58). Group 4 TAS mmol measurements were not different at baseline, 24, 48, 72, and 96 hours (P=0.63). It was determined that TAS mmol measurements at baseline, 24, 48, 72, and 96th hours differed according to the groups. It was found that TAS mmol measurements at baseline, 24, 48, 72, and 96 hours were lower in group 3 than in groups 2 and 4 (P=0.01) (Figure 3).

In the study, it was determined that group 1 TOS mmol H_2O_2 measurements were not different at baseline, 24, 48, 72, and 96 hours (P=0.24). Group 2 TOS mmol H2O2 measurements were not different at baseline, 24, 48, 72, and 96 hours (P=0.17). Group 3 TOS Mmol H_2O_2 measurements were not different at baseline, 24, 48, 72 and 96 hours (P=0.08). Group 4 TOS mmol H_2O_2 measurements were found to be different at baseline, 24, 48, 72, and 96 hours (P=0.01). The reason

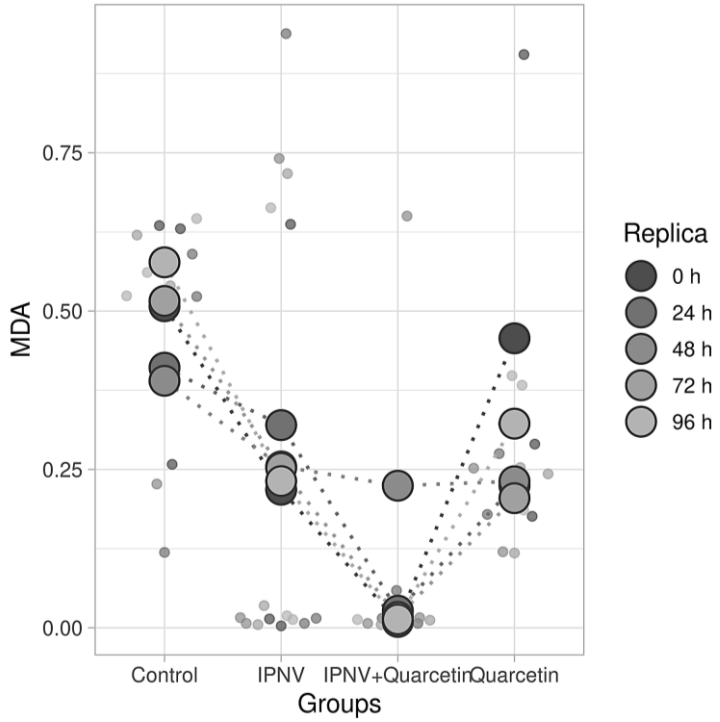

Figure 2. Results of MDA µ /l measurements between groups and according to measurement hours.

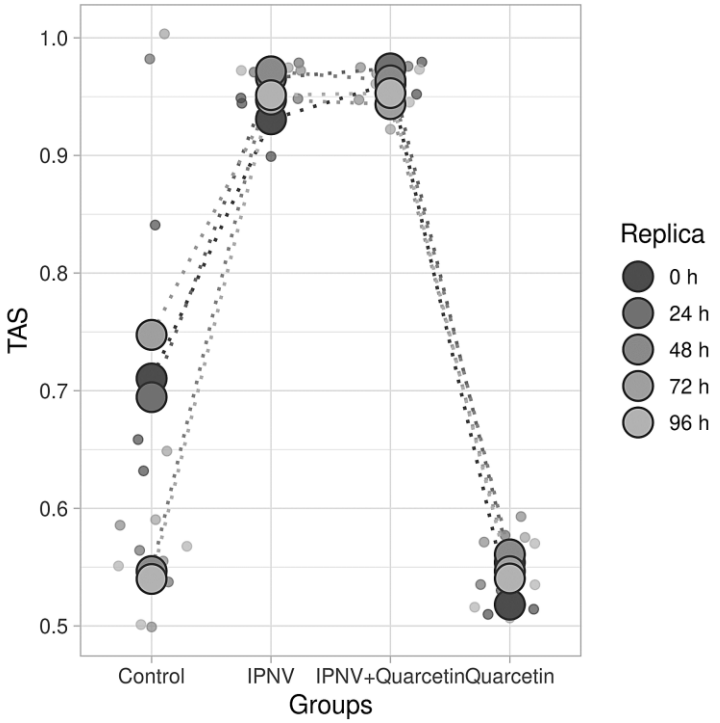

Figure 3. Results of TAS mmol Trolox Measurements Between Groups and According to Measurement Hours.

for the difference was found to be higher at 96 hours compared to all other hours. It was determined that TOS mmol H_2O_2 measurements at baseline, 24, 48, 72, and 96th hours differed according to the groups. It was determined that TOS mmol H_2O_2 measurements at baseline, 24, 48, 72, and 96 hours were higher for groups 3 and 2 compared to groups 1 and 4 (P=0.01) (Figure 4).

In the study, it was determined that there was no statistical difference in group 1 ceruloplasmin mg/dL measurements at baseline, 24, 48, 72, and 96 hours (P=0.13). There was no statistical difference in Group 2 and Group 3 ceruloplasmin mg/dL measurements at baseline, 24, 48, 72, and 96 hours (P=0.08). Group 4 ceruloplasmin mg/dL measurements were not statistically different at baseline, 24, 48, 72, and 96 hours (P=0.13). It was determined that ceruloplasmin mg/dL measurements at baseline, 24, 48, 72, and 96th hours did not differ according to the groups. It was determined that ceruloplasmin mg/dL measurements at baseline, 24, 48, 72, and 96 hours were at similar levels in groups 1, 2, 3, and 4 (P>0.05) (Figure 5).

In the study, it was determined that group 1, 2, 3, and 4 GPx activity µm/L measurements were not different at baseline, 24, 48, 72, and 96th hours (P=0.05) (P=0,33) (P=0,38) (P=0,10). It was determined that the initial, 24, 48, 72, and 96th hour GPx activity µm/L measurements performed in the study did not differ according to the groups. It was determined that GPx activity µm/L measurements measured at baseline, 24, 48, 72, and 96th hours were at similar levels in groups 1, 2, 3, and 4 (P>0.05) (Figure 6).

Discussion

IPNV causes an infectious disease characterized by pancreatic necrosis in different fish species and is associated with high mortality (Wolf, 1988). In addition, the IPN virus has a worldwide spread and can be isolated from almost all European countries (Reno, 1998). The presence of IPNV in fish is reported from Türkiye and many other fish-producing countries in the world. Enterprises facing IPNV infection cause both serious economic losses and some restrictions on international trade (Gürçay et al., 2013). Quercetin, which is an important flavonoid and commonly found in foods in the form of aglycones or glycosides, was effective in binding viral proteins and showed antiviral activity *in vitro*. It has also been reported to reduce susceptibility to viral infection (Davis et al., 2008) by protecting from oxidative stress (Kumar et al., 2003). IPNV, which is very important for fish farming and the economy, is known to be an important source of infection for the fish industry. In this study, it was determined that quercetin, which is a plant-derived flavonoid, helps to terminate lipid peroxidation chain reactions and minimize the damage caused by free radicals occurring in the cell, can provide an important development for fish farming and economy, and the maximum nontoxic dose of quercetin is 50 µmol. Cheng et al. (2005) examined different quercetin concentrations (0.1, 1, 10, 25, 50, and 100 µmol/L) in HepG2.2.15 (hepatocellular carcinoma) and HuH-7 cells and at different time intervals (2, 4, and 6 days) using Cell Counting Kit-8 (CCK-8) and determined

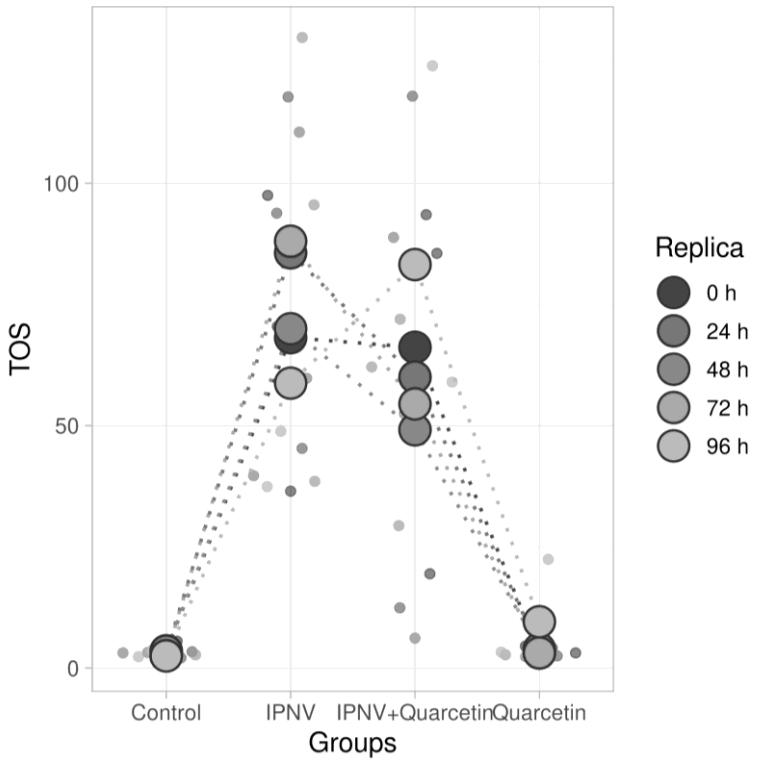

Figure 4. Results of TOS mmol H_2O_2 Measurements Between Groups and According to Measurement Hours.

that quercetin was noncytotoxic in both cells at the concentrations tested. Moreover, the maximum treatment concentration was chosen as 50 µmol/L (Cheng et al., 2015). In two other studies on the subject, it was reported that quercetin had very low toxicity and the half maximal (50%) cytostatic concentration (CC50) was more than 100 µmol/L (Kelly, 2011; Romero et al., 2005). When the result of our study was examined, it was determined that it was compatible with the results of the studies.

It has been reported that IPNV enters the host through the digestive tract, gills, and lateral lines and spreads through the blood to the pancreas, liver, and spleen (Smail & Munro, 1985). The immune system is the first line of defense against viral pathogens in fish and limits viral replication (Magnadottir, 2010). Fish antibodies are produced by B lymphocytes and neutralize virus particles by adhering to their surface, initiating immune system activation, and taking part in the proliferation and activation of lymphocytes

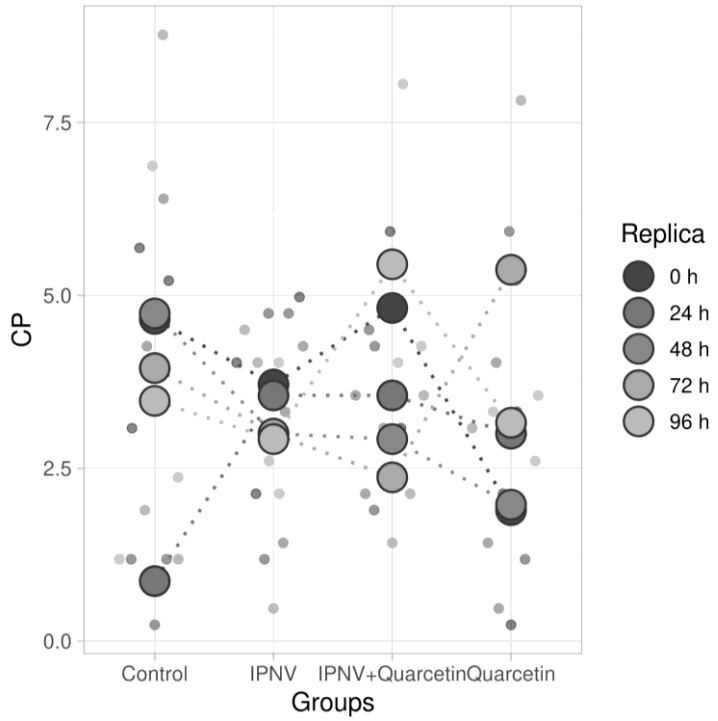

Figure 5. Results of Ceruloplasmin mg/dl Measurements Between Groups and According to Measurement Hours.

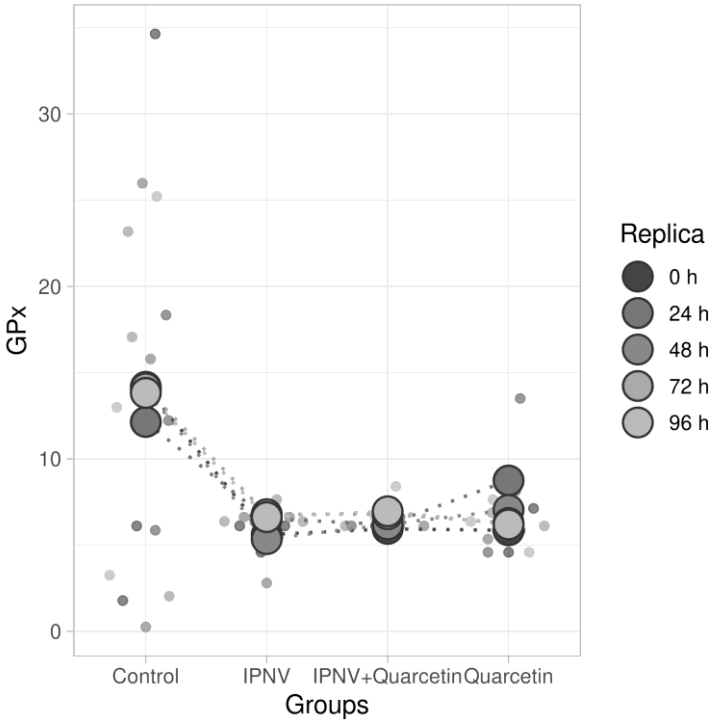

Figure 6. Results of GPx activity µm/l Measurements Between Groups and According to Measurement Hours.

(Durmaz, 2016). In addition to the immune system, fish also have an antioxidant defense system. Studies in both mammals and fish have shown that oxidative stress has many effects on immunity (Öğüt, 2005). In a study on the mechanism of viral replication in cells, the relationship between virus and host was examined and it was concluded that there is an important relationship between reactive oxygen species released from the host and viral pathogenesis. It is emphasized that O_2 production increases as an immune response in the host after viral infection and these oxidant molecules may be pathogenic molecules in the formation of viral diseases (Akaike et al., 1998). Phagocytosis, which constitutes the first step of cellular defense, is usually carried out by macrophages and neutrophils in fish (Ocak, 2006). During phagocytosis in the cell, oxidants such as H_2O_2 are formed as a result of the respiratory burst. Although hydrogen peroxide is not a free radical, it reacts with a transition metal (Fe^{+2}) to produce free radicals and can enter the cell through the cell membrane. It is stated that all of these compounds formed by the defense system against viruses are oxidative substances that damage the organism. The GPx enzyme catalyzes H_2O_2 and reduces it to water and non-toxic alcohols (Biller-Takahashi & Urbinati, 2014).

Antioxidants play an important role in the treatment of viral diseases. In a study, quercetin used at different concentrations (0.1, 1, 10, 25, 100 µmol/L) was examined in hepatocellular carcinoma and HUH-7 cells at different time intervals and it was reported that the concentrations tested in both cells were noncytotoxic. It was also noted that quercetin reduced viral replication when used at concentrations of at least 10 µmol/L and higher. Different concentrations of quercetin were applied to IPNV-infected cells and it was observed that there was a 5% decrease in IPVN viral load after 10 µmol/L quercetin application, 35% in cells treated with 20 µmol/L quercetin, and 40% at 50 µmol/L (Cheng et al., 2015). In our study, the viability of cell cultures treated with 50 µmol quercetin was 5 2% at the end of 24 hours and 50% after 48 hours, while the viability of cells treated with 100 µmol quercetin was 47% at the end of 24 hours and 49% after 48 hours. When we compared the virus-infected group with the quercetin-added group in our measurement results, it was determined that quercetin was effective in reducing the oxidative effect of IPNV on the cells both within the group and between hours. In a study with flavonoid antioxidants, structure-activity relationships were examined and it was determined that flavonoids containing hydroxyl in the ortho-position were more effective in scavenging free oxygen radicals than flavonoids containing hydroxyl in the meta-position. It is argued that flavonoids containing a hydroxyl group in the ortho-position of the B ring have higher effects in scavenging free radicals (Zhang, 1999). In addition, it was determined that there was no statistical significance between groups and hours in GPx results. In MDA results, there was no significant difference between hours, but there were significant differences between groups.

According to the TOS results, it was determined that there was no statistically significant difference at 0, 24, 48, 72, and 96th hours for group 1, group 2, and group 3. When group 4 TOS results were analyzed, it was determined that there was a statistically significant increase in the 96th hour measurement result compared to the other measurement hours. In addition, according to the results of statistical analysis between the groups, group 2 and group 3 results were significantly higher than group 1 and group 4. According to the results of TAS analysis, it was determined that there was no statistically significant difference for group 1, group 2, group 3, and group 4 at 0, 24, 48, 72, and 96th hours. According to the results of statistical analysis between the groups, it was determined that the measurement results of group 3 were significantly lower than group 2 and group 4. This shows that quercetin reduces oxidation as an antioxidant. It is thought that quercetin in the medium cleans oxidants and thus reduces the activation of TAS.

According to the MDA results, it was determined that there was no statistically significant difference at 0, 24, 48, 72, and 96th hours for group 1, group 2, and group 4. When the MDA results of Group 3 were analyzed, it was determined that there was a statistically significant increase in the 48th hour measurement result compared to the other measurement hours. According to the results of statistical analysis between the groups, there was no significant difference. According to the results of GPx analysis, it was determined that there was no statistically significant difference when 0, 24, 48, 72, and 96th hours were compared for group 1, group 2, group 3, and group 4. It was also determined that there was no significant difference according to the results of statistical analysis between the groups. According to the results of ceruloplasmin analysis, it was determined that there was no statistically significant difference when 0, 24, 48, 72, and 96th hours were compared for group 1, group 2, group 3, and group 4. In addition, according to the results of statistical analysis between the groups, it was determined that there was no significant difference. While there was no statistical difference in GPx activity and ceruloplasmin levels in the virus-infected and quercetin groups, when we examined the differences between the hours, it was determined that the ceruloplasmin level decreased in the IPNV group compared to the 0th hour, while the ceruloplasmin level increased in the IPNV+quercetin group. The increase in ceruloplasmin level gives a clue about the detection of the disease in the cell. As a result of our ceruloplasmin measurement, when the control group and virus-infected groups were compared, it was determined that ceruloplasmin levels increased in the IPNV and IPNV+quercetin groups. At the 0th hour, when we examined the differences between the groups, it was observed that the ceruloplasmin level of the IPNV+quercetin group increased more than the IPNV

group. We think that the reason for this increase is due to the antioxidant properties of quercetin. In a study by Wang et al. (2020) on the effects of quercetin on immunity, antioxidant indices, and disease resistance, zebrafish with an average weight of 1.0 ± 0.01 g were exposed to 0.01, 0.1, 1, 10, 100, and 1000 µg/L quercetin for 8 weeks, while control fish were exposed to only 0.01% DMSO. MDA contents were significantly lower at 1 and 10 µg/L quercetin exposure levels than in the quercetin-free group. However, no significant difference was observed in the other groups. The 10 µg/L quercetin groups showed significantly higher GPX activities than the quercetin-free group and 1000 µg/L groups (Wang et al., 2020). This showed that quercetin has a significant antioxidant effect. This effect may be due to the fact that this substance contains phenolic compounds. It has been reported that quercetin is a polyphenolic flavonoid compound, a free radical scavenger, and a powerful antioxidant (Rice-Evans et al., 1996). González-Esquivel et al. (2015), investigated the effect of quercetin on oxidative stress in the liver and kidney caused by titanium dioxide (TiO_2) nanoparticles in rats and reported that quercetin loaded with titanium dioxide (TiO_2) reduced MDA in liver and kidney and had a protective effect by increasing glutathione reductase levels (González-Esquivel et al., 2015). Robaszkiewicz et al. (2007) conducted *in vitro* cell studies and found that quercetin reduced ROS production in cells. They found that low concentrations of quercetin increased total antioxidant capacity after 6 hours of incubation, while high concentrations decreased both thiol content and TAS of cells. In cells incubated up to 6 hours with quercetin, no increase in glutathione S-transferase activity was detected, while a decrease in the activity of these enzymes was observed for high quercetin concentrations (Robaszkiewicz et al., 2007). It was observed that similar data were obtained with the results of this study and our study results and it was determined that our study results were compatible with the literature data.

Conclusion

In cells infected with IPNV in the laboratory environment, it has been observed that quercetin, a plant-derived flavonoid, can minimize the damage caused by scavenging free radicals in the environment and thus provides an important development for fish farming and economy. We also believe that *in vitro* studies can be a model for *in vivo* studies to evaluate systemically. We think that our study will contribute to new studies to be carried out for the prevention and treatment of viral diseases that are important in the aquaculture industry.

Ethical Statement

Not applicable.

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Author Contribution

First Author: Conceptualization, Writing, Investigation, Second Author: Supervision, Writing - review and editing, Data Curation, Formal Analysis

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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