The involvement of antioxidant, stress, and immune-related genes in the responsive mechanisms of common carp (*Cyprinus carpio*) to hypersalinity exposure

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Salinity stress is one of the marked influencing factors on the ecophysiology of aquaculture and is considered an important reason for the retreat of the fish industry. The current study is an endeavor to elucidate the molecular mechanisms that underlie the response to salinity stress in common carp. Fish (Average weight 5 ± 2 g) were randomly distributed into two groups; the 1st is a control was exposed to tap water (0.2 ppt salinity) and the 2nd is a treated was exposed to hypersalinity (10 ppt salinity) for five days. Serum biochemical indicators including total protein, albumin, globulins, A/G ratio, blood glucose, cortisone, Na^+, K^+, and Cl^- levels were evaluated. Besides, Tumor necrosis factor-α, interleukin-1β, corticotropin-releasing hormone, and catalase enzyme mRNA expression levels were assessed in lymphoid and immunocompetent organs (liver and spleen) and osmoregulatory organs (kidney and gills) by using Real-time qPCR. Hypersalinity adversely affected the biochemical markers; total protein, albumin, and globulins decreased significantly; however, blood glucose, serum cortisol, and sodium markedly increased in fish exposed to hypersalinity compared with the control. In addition, from the molecular point of view, all the evaluated genes were upregulated at a high expression rate in the liver compared with other studied organs after the salinity challenge. On the contrary, hypersalinity modulated the expression of immune-related genes (Tumor necrosis factor-α and interleukin-1β)

in the kidney and spleen and upregulated corticotropin-releasing hormone mRNA in all studied organs except gills. In conclusion, the obtained data clarified the molecular and biochemical mechanisms of salinity stress on the liver, kidney, spleen, and gills. Furthermore, it strongly suggests the implication of neural, endocrine, and immune systems in the responsive mechanisms to the salinity stress in carp.

1 Introduction

Aquaculture is one of the world's fastest-growing industries as 50% of fish production yield is achieved by fish farms (Fitzsimmons et al., 2015). However, the fish farming industry is facing a considerable challenge, in particular, water salinity and the scarcity of freshwater (FAO, 2003). This is owing to water shortage and climate changes including temperature elevation, which directly impacts water evaporation and, subsequently, higher salinity concentrations. Besides, the freshwater ecosystem is polluted by anthropogenic salts derived from industrial and agricultural waste (Velasco et al., 2019). In addition, salinity has a marked impact on the ecophysiology of fish and is considered an essential ecological factor in the retreat of the fish industry. Moreover, salinity change is the main cause of deleterious effects on physiological hormones and enzyme activities, reflecting fish bioactivity and survival (Cao et al., 2022). Furthermore, hypersalinity stress is considered the harshest aquatic environment disturbing the physiological functions of growth performance, metabolic rate, food intake, and hormonal stimulation in aquaculture (Gonzalez, 2012).

Common carp (*Cyprinus Carpio*) is a stenohaline freshwater fish with a low endurance ability to salinity. Salinity is one of the common causes of ecosystem disturbance and physiological homeostasis imbalance, ending in high mortalities of carp due to osmotic shock (Evans and Kültz, 2020). Research has shown that when salinity is elevated, homeostasis is achieved by increasing the concentrations of Na+ and K+ in the blood, and there is enhanced activity of gills Na⁺/K⁺ ATPase (Salati et al., 2011). Moreover, Alkhshali and Al-Hilalli (2019) reported increases in RBCs, HB, PCV, and WBCs in common carp in response to steady elevations in the salt concentrations (from 5 to 10 to 15 g/L) as a physiological response to environmental salinity perturbations (Al-Khshali and Al Hilali, 2019). In addition, the chronic salinity change contributes to the disturbance of liver function in common carp (Alkhshali and Al-Hilalli, 2019). The adverse effect of salt stress was extended to general performance in common carp as it reduced food intake and growth rates with unfavorable effects on survivability after that, with depletion of both muscle and liver glycogen ending with energy expenditure (Sharma et al., 2020). Furthermore, drastic salinity change has been associated with oxidative stress and the production of reactive oxygen species (ROS) in fish accompanied by suppressed immune functions and antioxidant defense abilities (Kim and Kang, 2015).

Exposure to salinity stress is associated with genetic response and the modulation of gene expression in hypersalinity tolerance. For instance, the function of *Na+/K+ ATPase* (NKA) has been studied in the gill and kidney of multiple species of fish including silver catfish (Marx et al., 2022), Nile tilapia (*Oreochromis niloticus*) (Mohamed et al., 2021), Japanese eel (Tang et al., 2012), Senegalese sole (Armesto et al., 2015), and pufferfish (*Tetraodon nigroviridis*) (Wang et al., 2017). Since *NKA* is in the gills, increased expression of NKA in gills after 14 days of exposure to 10 ppt saline is probably the result of the excretion of ions that flow into the body across the thin epithelia of the gills. Also, aquaporins are water channels that play a key role in water transportation in hypersalinity exposure. The gene expression of aquaporin 3 is upregulated in gills 6 days after transfer from 5 ppt to 30 ppt saline in pufferfish. Moreover, the change in environmental salinity (10 and 25%) enhanced immune response by the elevation of mRNA expression profiles of cytokine genes *IL-1β, IL-6, IL-10*, and *TNF-α* in coastal fish Scatophagus argus (Lu et al., 2022).

As an adaptive and hemostasis response from the fish against salinity challenge, antioxidant enzymes, immunostimulants (pro-inflammatory cytokines), stress hormones, and non-specific immunity are elevated (Tapia-Paniagua et al., 2011). Therefore, evaluating these parameters in fish is an appropriate marker for measuring the potential influence of higher salinity exposure on innate immunity and antioxidant tolerance (Saurabh and Sahoo, 2008).

The hypothesis of the current study is to explore the molecular and biochemical mechanism by which the common carp respond to the hypersalinity and change in ecosystem. Furthermore, it is expected that the fish being studied brings about the change in the disturbed environment through homeostasis by modulating the gene expression of proinflammatory cytokines (*TNF* and *IL-1β*), *stress hormones (CRH) and antioxidants (*CAT*) in the* osmoregulatory and immune organs, mainly the liver, kidney, spleen, and gills. In addition, the molecular mechanism that explains the adaptation of common carp to hypersalinity is elusive; therefore, the present study is designed to bridge the gap between hypersalinity and homeostasis through molecular and biochemical mechanisms.

Although many studies have manipulated the effect of hypersalinity on common carp, molecular analysis is still elusive. The

current study is conducted to figure out the ability of common carp – depending on their economic importance – to acclimate to hypersalinity, especially in the harshest change in ecosystem conditions such as freshwater salinization, pollution, and water scarcity problems

Subsequently, the current study endeavors to investigate the physiological mechanisms depending on the molecular and biochemical aspects in the rescue of the disturbed ecosystem due to the change in environmental salinity. The biochemical markers including total protein, albumin, globulins, A/G ratio, blood glucose, cortisone, Na^+, K^+, and Cl^- levels were identified. Furthermore, the mRNA expression of pro-inflammatory cytokines (*TNF* and *IL-1β*), corticotropin-releasing hormone (*CRH*; a stress hormone), and catalase enzyme (*CAT*; an antioxidant marker) were assessed in the gills, liver, kidney, and spleen.

2 Materials and methods

2.1 Fish management

Common carp (*Cyprinus carpio*) were obtained from a commercial fish farm with an average weight of (5 ± 2 g). The fish were acclimated for 3 weeks under laboratory conditions in the Department of Fish Diseases and Aquatic Laboratory of the Faculty of Veterinary Medicine, South Valley University, Qena, Egypt, in accordance with the guidelines on the care and use of experimental animals for scientific purposes set by South Valley University. During the acclimation period, the water temperature was kept at 22℃, the dissolved oxygen at 6.1 ± 0.5 mg/L, and the pH at 7.1. The fish were fed a commercial diet (Table 1) twice daily at 3% of their body weight, maintained on a 12 h:12 h light/dark cycle, and reared in a porcelain tank re-circulation system ($260 \times 65 \times 70$ cm) provided with pump aeration and filled with fresh dechlorinated water (salinity = 0.2 ppt).

2.2 Experimental design

The fish were randomly distributed into two experimental groups (18 fish for each) and stocked in six fiberglass aquaria ($60 \times 30 \times 40$ cm) containing 120 L (six fish per tank in triplicates) of dechlorinated tap water.

One control group was kept in dechlorinated tap water (salinity = 0.2 ppt) and another salinity-exposed group was reared at 10 ppt for 5 days. The salinity is obtained by mixing an adequate amount of commercial-grade NaCl with dechlorinated tap water. Water salinity levels were gradually raised in the experimental tanks by adding NaCl to water at 3 g/l daily until reaching the proposed levels of salinity and the concentration was selected after the preliminary test run using a salinometer to determine salinity concentrations (Thermo Electron corporation model Orion 150 A+).

The water quality indices (temperature, pH, dissolved oxygen, and nitrite) were checked daily during the study using Martini Instruments Model 201/digital to detect the temperature, dissolved oxygen, salinity, and pH. The total ammonia level was detected calorimetrically using a HI-83303–02 Aquaculture Multi-parameter Photometer (Woonsocket, RI, USA). The aquarium was kept at 20 ± 1°C under natural light cycle conditions. The fish were fed twice a day with commercial floating powder feed containing 45% protein at a feeding rate of 3% of their body weight. During the experimental period, fish were checked twice a day for uncommon clinical symptoms and mortality rates (Emeish, 2019).

2.3 Sample collection

Fish were euthanized using 0.033 mL/L eugenol (Hamáčková et al., 2006) (clove oil) on the 5th day after exposure to salinity stress. Blood samples were collected in Eppendorf tubes from the caudal vein by severing the tail and centrifuging at 3000 rpm for 15 min. The collected sera were stored at -20°C until they were used in biochemical assays. The liver, kidney, spleen, and gills (30 mg/ organ) were collected from control and salinity-exposed groups and stored in RNA later (Omega BIO-TEK, GA, USA) at −80°C until quantitative qPCR gene expression analysis.

2.4 Biochemical assessment

All biochemical parameters were monitored spectrophotometrically according to standard methods using commercial laboratory kits. Serum total proteins, albumin, sodium, and potassium chloride kits (Spectrum, Egyptian Company for Biotechnology, Obour City, Egypt) were used. Globulin level (g/dl) was calculated mathematically by subtracting albumin from the total protein value. In addition, blood glucose levels in whole blood samples were checked using the Gluco Dr. auto blood glucose monitoring system and gold-plated test strips (All Medicus Co., Ltd., Germany) following the manufacturer's description. All biochemical parameters were carried out by using T80 UV/VIS spectrophotometer (PG Instruments, UK). Serum cortisol levels were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (#CAN-C-270; Diagnostics Biochem Canada Inc., Canada). A cortisol assay was done by a microplate reader (Infinite 50, Männedorf, Switzerland) at a wavelength of 450 nm according to the manufacturer's instructions.

TABLE 1 Formulation and proximate composition of basal diets.

S	Ingredient	g/100 g
1	Yellow corn	34.9
2	Vegetable bean meal	28.6
3	Fish meal	17.0
4	Wheat bran	9.3
5	Vegetable oils	6.5
6	Mineral mixture	1.7
7	Vitamin mixture	1.0
8	Ground limestone	0.7
9	Bone meal	0.3

2.5 Gene transcription

Total RNA was extracted from the liver, kidney, spleen, and gill tissues using RNeasy® Mini kit (QIAGEN, Germany) following the manufacturer's instructions, and the quantity of the RNA was estimated by using Nanophotometer (NanoDrop Technologies, Wilmington, USA). For cDNA synthesis, 1μg of each RNA sample was reverse transcribed to cDNA in the Vetiti™ 96 Well Thermal Cycler, according to the instructions using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, MA, USA).

RNA expressions of *TNF-α*, *IL-1β*, *CRH*, and *CAT* mRNA were measured using a real-time qRT-PCR. The cDNA products were amplified using the HERA^PLUS SYBR® Green qPCR Kit (Willowfort, England), utilizing a Stratagene Mx3005p® real-time qPCR detection system (Agilent Technologies, MA, USA). Each 20 μL reaction mixture contained 10 μL Sybr Green Master Mix, 1.5 μL cDNA, the gene-specific primers (0.5 μM each), and 1 μL of each primer (10 μM of forward and reverse) of the proposed genes (Table 2), and the volume was completed up to 20 μL with sterile nuclease-free PCR grade water, and no template PCR control was included. The cycling conditions were the initial enzyme activation step at 95°C/3 min followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. To confirm the amplification of specific products, cycles were continued to check the melting curve.

Each sample was normalized to the expression level of the housekeeping genes Beta-Actin (*β-actin*) and Elongation Factor 1-α (*EF1α*) to correct for differences in template input and reverse transcriptase efficiencies. All reactions were assayed in duplicate on triplicate samples, and the threshold cycle (Ct) value was obtained from the automatic setting on the Mx3005p® real-time qPCR detection system and calculated. The fold change of target gene expression in a target sample relative to a reference sample, normalized to a reference gene. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.6 Statistical analysis

All data were analyzed with GraphPad Prism software (GraphPad 8.0.1 Software, San Diego, CA, USA) using the student's *t-test*. Data were expressed as the mean ± standard error, with significant differences defined as p < 0.05. RStudio (R version 4.0.2) was used to generate the correlation heatmap between variables, clustering heatmap among different treatments, and the variable important projection. The current data were validated for normality and homogeneity of variance using the Shapiro-Wilk normality test and the Kolmogorov-Smirnov normality test.

3 Results

3.1 The effect of salinity on mortality records

The mortality rate was recorded in the 0.2 and 10 ppm salinity-exposed fish during the experimental period. Fish exposed to 0.2 ppm groups survived during the experimental period with a mortality rate of 0%; however, those challenged with 10 ppm disclosed different mortality rates; for instance, the death percentage on the 3rd day was 11.11%, was 16.66% on the 4th day, and was 33.33% on the 5th day, as shown in Figure 1. These data suggest the existence of a negative impact of hypersalinity medium on fish survival rate.

3.2 The effect of salinity on biochemical indices

The hypersalinity-exposed fish denoted significant (P < 0.05) decreases in the serum levels of total protein, albumin, and globulins when compared with the corresponding control, as depicted in Figures 2A–C, respectively. Conversely, the A/G ratio elevated non-significantly after salinity exposure compared with control fish (Figure 2D). Furthermore, blood glucose and serum cortisol levels markedly (P < 0.05) increased after exposure to salinity stress when compared with control (Figures 2E, F, respectively). As a response to the change in the salinity environment, circulating Na^+, K^+, and Cl^- exhibited remarkable (P < 0.05) increases compared with the corresponding controls (Figures 2G-I, respectively). These findings proposed the effect of salinity exposure on the biochemical parameters measured in this study.

TABLE 2 The forward and reverse primers for targeted genes.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Accession No	References
CRH	CATCCGGCTCGGTAACAGAA	CCAACAGACGCTGCGTTAACT	AJ317955	(Bernier et al., 2012)
CAT	AGACGACACCCATCGCTGTTCG	AAGGTCCCAGTTGCCCTCATCG	GQ376154.1	(Ghelichpour et al., 2019)
IL-1β	AAGGAGGCCAGTGGCTCTGT	CCTGAAGAAGAGGAGGCTGTCA	AB010701.1	(Sunarto and Mccoll, 2015)
TNF-α	GAGCTTCACGAGGACTAATAGACAGT	CTGCGGTAAGGGCAGCAATC	JF957372.1	(Sunarto and Mccoll, 2015)
β-actin	GATTCGCTGGAGATGATGCT	GATGGGGTACTTCAGGGTCA	M24113	(Schyth et al., 2006)
EF1α	GGAGCCCAGCACAAACATG	TTACCCTCCTTGCGCTCAAT	AF485331	(Mráz, 2012)

CRH, corticotropin-releasing factor; *IL-1β*, interleukin-1beta; *TNF-α*, tumor necrosis factor-alpha; *CAT*, catalase; *EF1α*, elongation factor1-alpha.
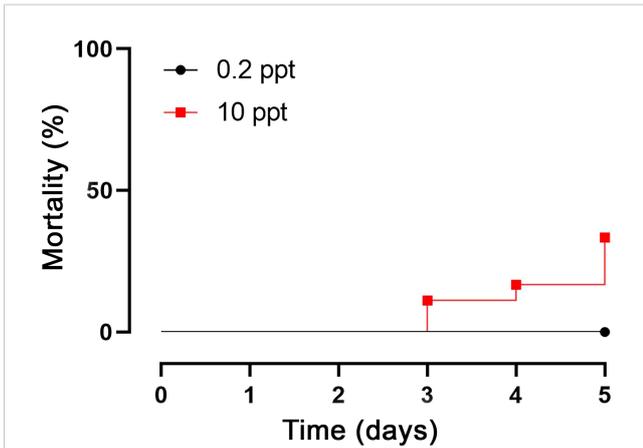
FIGURE 1
Mortality rates of experimental fish after 5 days' exposure to 0.2 and 10 ppt salinity.

3.3 The effect of salinity on stress, inflammation, and antioxidant gene expressions

Figure 3 shows the expression of the studied genes in liver tissue after exposure to 0.2 (Control) and 10 ppt for 5 days. The liver in salinity-exposed fish presented a marked ($P < 0.05$) upregulation of *TNF-α, IL-1β, CRH*, and *CAT* mRNA expression levels when compared with the control. Furthermore, the kidney and spleen of common carp exposed to salinity 10 ppt revealed dramatic ($P < 0.05$) upregulations of *TNF-α, IL-1β*, and *CRH* mRNA expression in comparison to controls. However, both organs revealed slightly increased mRNA expressions of antioxidant markers (*CAT*) compared to controls.

In gills tissue, the mRNA expression of *IL-1β* revealed a significant ($P < 0.05$) enhancement in the high salinity-exposed
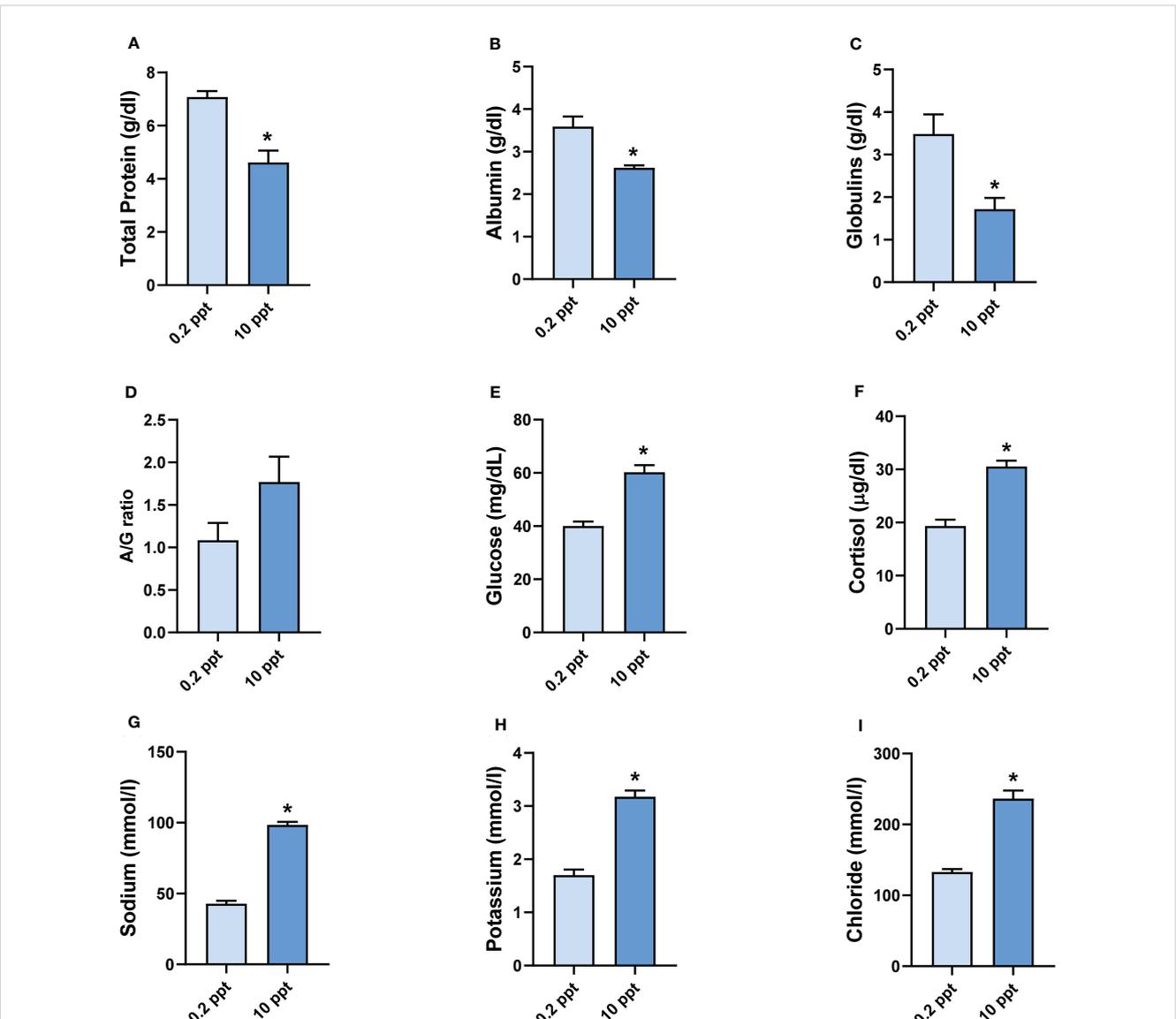

FIGURE 2
Biochemical analysis in common carp after exposure to 0.2 and 10 ppt salinity. **(A)** Serum total protein level (g/dl). **(B)** Serum albumin level (g/dl). **(C)** Serum level of globulins (g/dl). **(D)** A/G ratio (%). **(E)** Blood glucose level (mg/dl). **(F)** Serum cortisol level (µg/dl). **(G)** Serum level of sodium (mmol/l). **(H)** Serum level of potassium (mmol/l). **(I)** Serum level of chloride (mmol/l). Data are expressed as mean $\pm$ SEM. *$P < 0.05$ vs. control group.
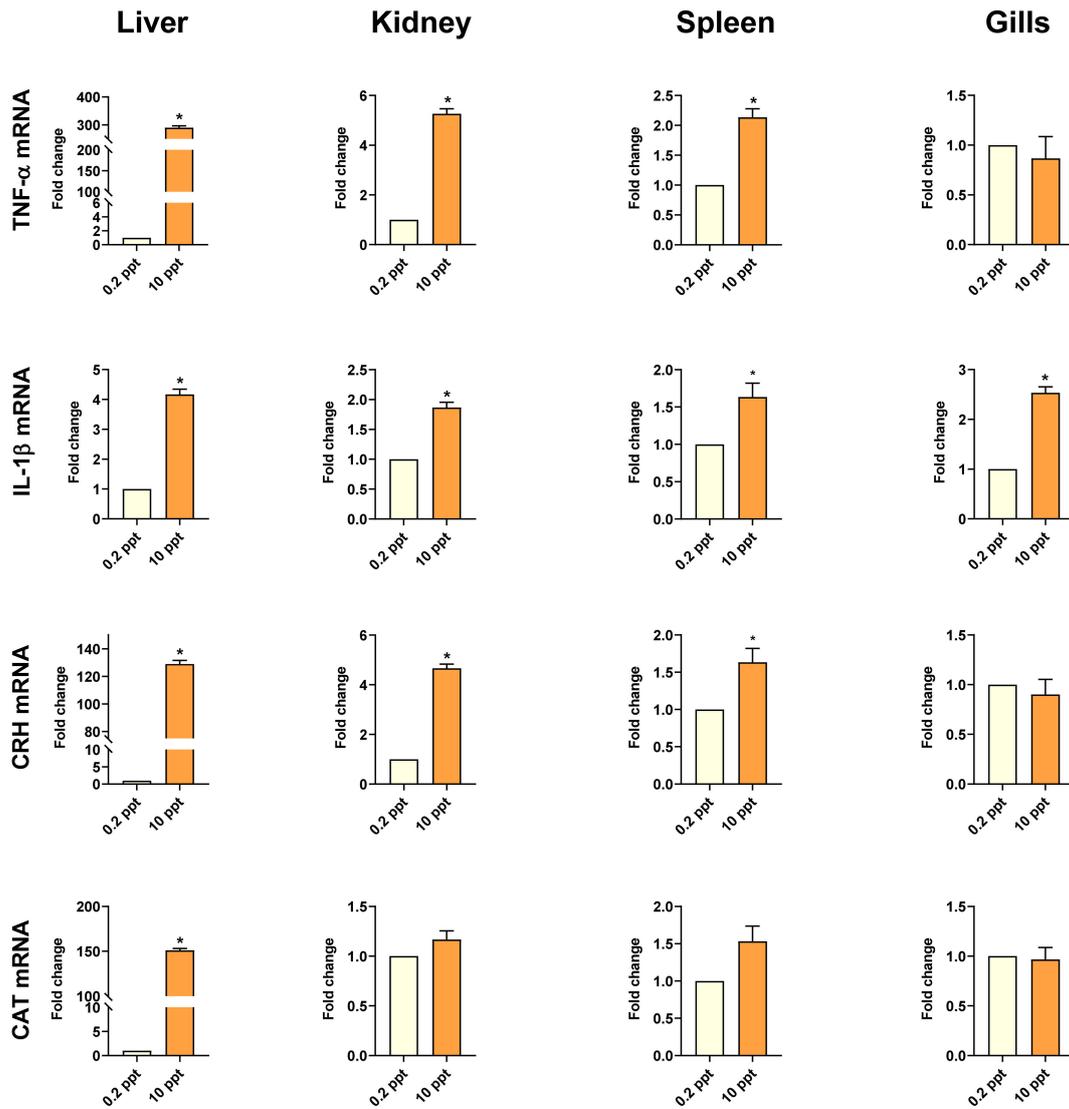
FIGURE 3
Cytokines (*TNF-α* and *IL-1β*), *CRH*, and *CAT* gene expression (relative to *β-actin* and *EF1α*) in common carp's liver, kidney, spleen, and gills exposed to 0.2 and 10 ppt salinity. Data are expressed as mean ± SEM. *$P < 0.05$ vs. control group.

fish in comparison with the control group. In contrast, the mRNA expression of *TNF-α*, *CRH*, and *CAT* showed a non-significant change (Figure 3).

The results in Figure 4 summarize the expression of proposed genes in the studied organs. *TNF-α* mRNA was markedly upregulated ($P < 0.05$) in the liver compared with the kidney, spleen, and gills. At the same time, the expression of the same gene revealed no significant elevation in the kidney compared with splenic and gill tissues, and the spleen compared with gills. Concerning *IL-1β* gene expression, the highest expression was exemplified in the liver, which showed a non-significant upregulation compared with other proposed organs. Furthermore, *IL-1β* gene expression slightly increased in the gills compared with the liver and spleen. Similar to *TNF-α*, *CRH* gene expression recorded a significant ($P < 0.05$) upregulation in the liver compared with the expression of the other three organs.

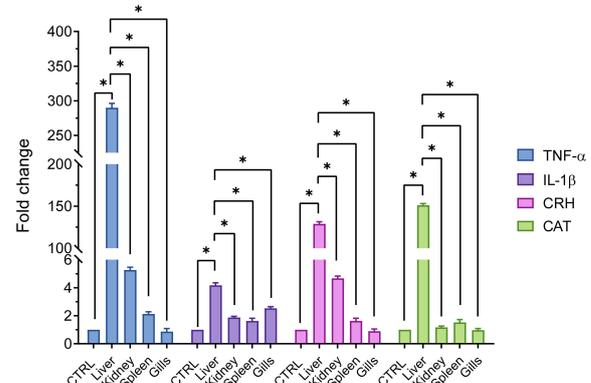**FIGURE 4**
Comparison of gene expressions (relative to *β-actin* and *EF1α*) in common carp's different organs (liver, kidney, spleen, and gills) exposed to 0.2 and 10 ppt salinity. Data are expressed as mean ± SEM. *$P < 0.05$ vs. control group.
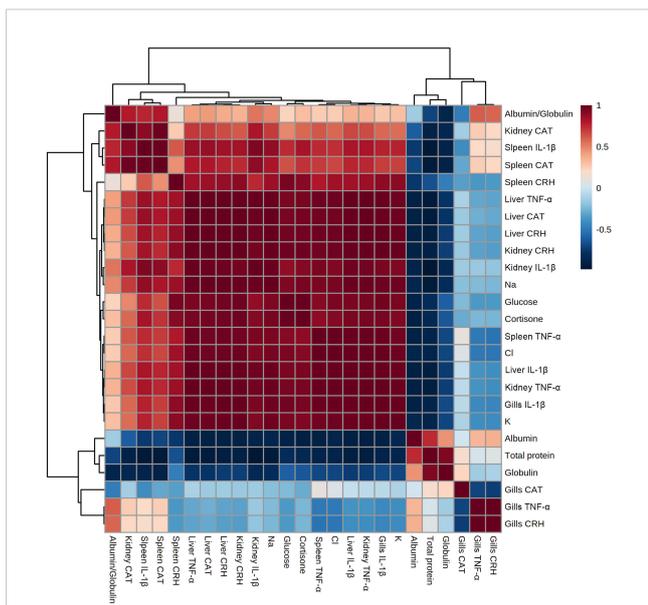
FIGURE 5
Correlation heatmap of all variables. The clustering heatmap provides an intuitive visualization of all data sets which summarizes the correlation between all measured biochemical variables and mRNA expression of target genes in response to 0.2 and 10 ppt salt concentrations for 5 days in each sample. The contribution is explicated by a colored scale ranging from the highest (red indicates positive correlation) to the lowest (blue indicates negative correlation). The degree of correlation is shown by the color's intensity. The various squares are colored in accordance with Pearson's correlation coefficient between all measured variables.

Furthermore, the former gene increased non-significantly in the renal tissue by three and five folds compared to the spleen and gill expression. Finally, *CAT* gene expression revealed prominent and significant ($P < 0.05$) upregulations in the hepatic tissue compared with the renal, splenic, and gill tissues, which expressed almost the same values of the *CAT* gene. The abovementioned gene expression data indicated that the recorded gene alterations were organ dependent.

3.4 Hierarchical clustering heatmap and variable important project (VIP) score

The clustering heatmap depicted in Figure 5 provides an intuitive visualization of all datasets which summarizes the correlation between all measured biochemical variables and mRNA expression of target genes in response to 0.2 and 10 ppt salt concentrations for 5 days. The contribution is explicated by a colored scale ranging from the highest (red indicates positive correlation) to the lowest (blue indicates negative correlation). The degree of correlation is shown by the color's intensity. The various squares are colored in accordance with Pearson's correlation coefficient between all measured variables. These data exhibit strong positive correlations between stress, proinflammatory cytokines, and antioxidants-expressed genes in all targeted tissues and the exitance of negative correlations between those genes and the total protein, albumin, and globulin concentrations in blood. In addition, Figure 6A summarizes the variable averages in response to
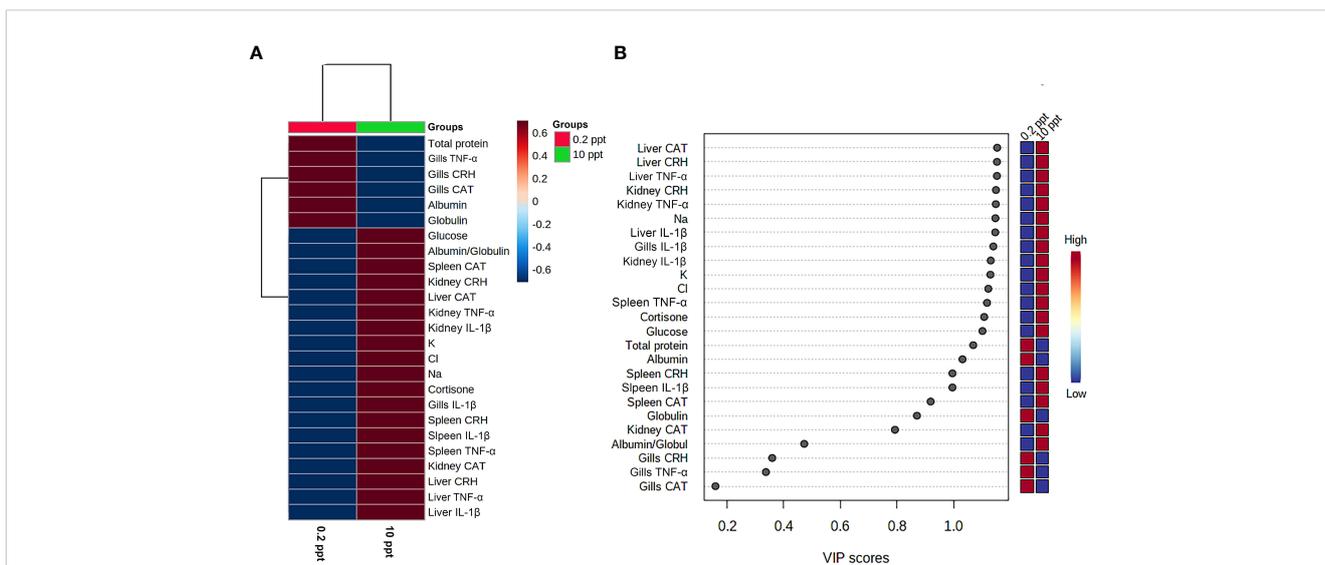

FIGURE 6
Clustering heatmap and variable importance in projection (VIP) scores. **(A)** Hierarchical clustering heatmap enables intuitive visualization of all data sets. Each colored cell on the map denotes the concentration values, with variable averages in rows and different treatment sets in columns. Dark red is the highest value on the gradation scale, and blue represents the lowest value. **(B)** Variable importance in projection (VIP) scores: the colored boxes on the right display the relative concentrations of the relevant measured parameters in each study group, while the contribution intensity is indicated by a colored scale spanning from the highest (red) to the lowest (blue).

different salinity concentrations in the current study. These data suggest the occurrence of physiopathological alterations in salinity-stressed fish.

Next, in order to reveal the most influencing variables in response to salinity exposure, the VIP score was calculated. As presented in Figure 6B, *TNF-α, IL-1β, CRH*, and *CAT* mRNA in liver, kidney, and spleen tissue along with blood Na$^+$, K$^+$, Cl$^-$, and cortisone strongly contributed to the mechanism involved in the salinity-induced physiopathological alterations in common carp.

4 Discussion

Salinity change is a major eco-stress factor affecting marine and estuarine organisms. However, many species can tolerate salinity fluctuation depending on how they cope. The current study is an in-depth understanding of the molecular mechanism response to salinity stress for 5 days by evaluating pro-inflammatory cytokines (*TNF-α* and *IL-1β*), the stress hormone (*CRH*), and antioxidant capacity (*CAT*) in the liver, kidney, spleen, and gills. Salinity challenge disrupts most physiological functions and biological processes, including hepatic, renal, and gill functions, blood dissolved gases, blood parameters, and hormonal regulation (Mohamed et al., 2021).

In addition, the tolerance to salinity stress is varied according to the aquatic species. In this study, mortalities started on the 3rd day and increased gradually until reaching 33.33% on the 5th day, indicating the limited ability of common carp to salinity adaptation. Therefore, the current study is necessary to shed light on the physiological mechanism depending on molecular and biochemical aspects in the rescue of the disturbed ecosystem and the change in environmental salinity. The current results are matching with that recorded by Akther et al., 2013 who found that acute exposure of *Barbodes gonionotus* to 15 ppt salinity showed a mortality rate of 20% after 24 h and 100% after 4 days.

The liver is the most affected organ by salinity stress, which is linked with a noticeable decrease in serum total protein, albumin, and globulins (Alkhshali and Al-Hilalli, 2019). The alteration of hepatic function is caused by oxidative stress and reactive oxygen species production (Kim and Kang, 2015), which is confirmed by the upregulation of *CAT* gene expression in the liver. Moreover, the change in circulating total protein, albumin, and globulin levels in the abovementioned results could be proof of water and electrolyte (dehydration) perturbation resulting from hypersalinity stress. In addition, the elevation of stress factors (*CRH* gene, cortisol, and blood glucose) in response to salinity challenges that are linked with the hypothalamic-pituitary-interrenal (HPI) axis often results in tissue modulation. Consequently, elevated corticosteroids reduce protein synthesis, enhance catabolism, and accelerate energy expenditure (Sopinka et al., 2016).

In addition, there was also a dynamic elevation in blood glucose levels. A stress reaction is expressed as increased blood glucose levels associated with high *CRH* gene expression and high circulating cortisol level, which is assumed to be a result of the hypothalamic-pituitary-interrenal (HPI) axis activity as evidenced by Wang et al., 2020. Interestingly, the elevation of blood glucose

levels after salinity exposure is accompanied by catecholamine secretion, which is part of the mechanism to counteract abrupt changes in environmental salinity. Furthermore, catecholamine release is followed by increased gill perfusion facilitating ion and gas exchange between fish bodies and the external environment (Demska-Zakęś et al., 2021).

The most influenced aspect after the salinity challenge is the ionic basis as common carp showed significant elevations of Na$^+$, K$^+$, and Cl$^-$. The primary organs for ion transport and water exchange in aquaculture are the gill epithelium, kidney, and intestinal epithelium (Evans and Claiborne, 2009). In this study, the elevation of Na$^+$ and Cl$^-$ is a preparatory stage for ionic equilibrium and water exchange by activating *Na$^+$/K$^+$ ATPase* and upregulating aquaporin3, especially in gills tissue (Jumah, 2020). The preceding studies denoted the elevation of Na$^+$, K$^+$, and Cl$^-$ in pikeperch exposed to high salinity, matching the current findings (Demska-Zakęś et al., 2021).

The liver is a fundamental organ that plays a crucial role in vital functions in living organisms, including metabolism, immunity, digestion, and detoxification (Atrees and Rabia, 2021). A stressful environment for aquatic organisms contributes to the deterioration of physiological cellular function by developing oxidative stress (Zhou et al., 2020). As a response to salinity stress, the present study demonstrates an immune response represented by the upregulation of *TNF-α* and *IL-1β* gene expression in hepatic tissue. The upregulation of these genes is a potent inflammation and pro-inflammatory response to regulate immunity and cope with stressors (Yahfoufi et al., 2018).

Furthermore, the results emphasized the upregulation of *CRH* gene expression in hepatic tissue. Oxidative stress induced by hypersalinity is associated with the activation of antioxidant enzymes involving *CAT* gene expression trying to overcome the adverse effect of ROS on DNA hepatocytes (Baldissera et al., 2019; Xavier et al., 2020).

The kidney is the second osmoregulatory organ after the gills, representing the crucial line (Buddington and Krogdahl, 2004). The osmoregulatory role of the kidney in stenohaline is prominent in environmental salinity stress. This role is represented in the excretion of a large amount of water through high glomerular filtration and activity reabsorption of the most ions to maintain fluid and electrolytes hemostasis (Takvam et al., 2021). *TNF-α, IL-1β, CRH*, and *CAT* monitored the molecular mechanism of renal tissue.

Like other organs, the kidney is affected adversely by hypersalinity, starting with the activation of pro-inflammatory cytokines (Brocker et al., 2012), supporting the current results by the elevation of *TNF-α* and *IL-1β* gene expression. In fish, the hypothalamic-pituitary-interrenal axis is a primary neuroendocrine modulatory system of immunity to tolerate stress (Gallo and Civinini, 2003). Moreover, it is stimulated by variable stressors *via IL-1β*, subsequently enhancing *CRH* release from the hypothalamic preoptic nucleus and cortisol hormone synthesis and secretion from inter-renal cells of the head kidney (Metz et al., 2006).

On the other hand, salinity stress has no effect on *CAT* gene expression in the kidney. According to Wang et al. (2016), antioxidant enzyme activity, including *SOD* gene expression, is

downregulated in juvenile marbled eels after a high salinity challenge but upregulated after a low salinity challenge. The fact that the relationship between enzymatic activity and mRNA expression is not linear (Chambers and Matrisian, 1997; Wang et al., 2016) explains the inexplicable changes in *CAT* gene expression.

The spleen is the main lymphoid and immunocompetent organ (Zapata et al., 2006). So, it is more sensitive and is rapidly affected by salinity stress, as shown in the present study; *TNF-α* and *IL-1β* gene expression is upregulated, agreeing with Xu et al. (2018) and Xu et al. (2020), who reported the upregulation of both genes in the spleen of *Tilapia nilotica* after exposure to 16% salinity. Similarly, *CRH* gene expression was upregulated in the spleen as a response to salinity stress. Harlé et al. (2018) confirmed that *CRH* receptors-2 are expressed in splenic B-cells, supporting our results. Likewise, other organs and the spleen suffer from oxidative stress after salinity stress, which is observed by enhancing antioxidant capacity through the upregulation of *CAT*.

The primary osmoregulatory organs are the gills (Hwang et al., 2018). They control ion transport *via* the Na^+/K^+ *ATPase* pump and water exchange *via* aquaporin channels (Li et al., 2006). Furthermore, *TNF-α* is the main pro-inflammatory cytokine associated with apoptosis and autoimmune conditions (Gao et al., 2015). The present results are consistent with El-Leithy et al. (2019), who confirmed the elevation of *IL-1β* gene expression in *Tilapia nilotica* after high salinity exposure. Moreover, salinity stress has a dual effect on immune response as it down-regulates or activates some immune mediators according to salinity dose and exposure time (Tort, 2011; Schmitz et al., 2017). In addition, some stress conditions induce salinity that can suppress or stimulate a specific pathway of cytokine production (Dhabhar, 2008). Furthermore, as reported by Castillo et al. (2008), cortisol secreted in response to stress has an influential inhibitory role in some cytokines, specifically *TNF-α* (Saeij et al., 2003).

Salinity stress is considered the hypothalamic-pituitary-interrenal axis activator represented by the neuroendocrine response that involves instant secretion of *CRH* by the preoptic nucleus of the hypothalamus, ACTH from the pituitary gland, and, finally, cortisol hormone from the head kidney (Tort, 2011). *CRH* allocation has been found in the common carp spleen and kidney, indicating that *CRH* receptors are highly expressed in these organs. Huising et al., 2004 explain that no change in *CRH* gene expression in gills tissue is due to a negative feedback mechanism. Hence, enhancing antioxidant enzyme activity can cause a disturbance in the balance between ROS production and removal, resulting in oxidative stress (Chowdhury and Saikia, 2020). Unexpectedly, the current study showed no change in gills *CAT* gene expression after exposure to salinity challenge due to no change in TNF-α. Pro-inflammatory cytokines (mainly TNF-α) trigger oxidative stress and stimulate the regulation and adaptation of the immune system to tolerate environmental stressors (Yahfoufi et al., 2018).

The activity of antioxidant enzymes including *CAT* after salinity challenge depending on the salinity dose and time of exposure to hypersalinity; antioxidant capacity has been improved in *Tilapia nilotica* after exposure to high salinity for a short time (Baldissera et al., 2019; Xavier et al., 2020). The current results agree with Xu

et al. (2018) who denoted that hypersalinity for a long time has no effect on *CAT* activity in *Tilapia nilotica*. In this study, the powerful upregulation of *CAT* gene expression in the liver may oppose its expression in the kidney, spleen, and gills *via* a negative feedback mechanism.

Hypersalinity induced an osmoregulatory modification in the gills including the activation of the Na^+/K^+ *ATPase* pump which is considered a compensatory response to the increased absorption of ions, so the elevation of gill's *NKA* activity is an integral part of a successful acclimation to increased salinity (Sangiao-Alvarellos et al., 2006) besides the high density of mitochondria in the gills and the elongation of the cristae and acquiring tubular profile. Subsequently, there is an increase in the ability to produce energy by ATPase synthase, which is necessary for the generation of ATP. Thus, high energy production is the key activator of the Na^+/K^+ *ATPase* pump which regulates ion exchange during hypersalinity ending with tolerance (Paumard et al., 2002). Moreover, the elevation of environmental salinity changes the ultrastructure of chloride cells which perform an integral role in acid-base regulation during exposure to high salinity by the regulation of Cl-/HCO3- exchange (Perry, 1997). Previous studies have proved the increase in the number and activity of chloride cells after salinity exposure in the gills of common carp (Al-Hilalli and Alkhshali, 2019), *Oreochromis mossambicus* (Lee et al., 2000), and juvenile Australian snapper (Fielder et al., 2007).

Exposure to high salinity requires a marked change in the osmoregulatory mechanism to maintain osmotic homeostasis by excessive water intake and ion excretion. For the active and passive transport of ions and water, all the epithelia in the intestine, gills, and kidney play an essential role in the exchange between water and ions, but the intestinal epithelia are predominant in the regulation of fluid intake among the previous organs (Evans, 2008). The intestine tight junction consists of several physiologically regulated proteins forming the circumferential seals around the adjacent epithelial cells called claudins. Claudins gene expression was upregulated during hypersalinity as they regulated the permeability of charged and uncharged molecules (Van Itallie et al., 2008).

The multivariate analyses represented by heatmaps and VIP scores affirm the abovementioned data. The clustering heatmap cleared observable differences between the concentration values of all variables in response to different changes in salinity % exposure. These data strongly confirm the responsive adaptive mechanisms of common carp to changes in salinity concentration. Therefore, the VIP scores revealed that the *TNF-α, IL-1β, CRH*, and *CAT* mRNA in the liver, kidney, and spleen tissue along with blood Na^+, K^+, Cl^-, and cortisone were the most contributing factors in the adaptive mechanism of common carp to salinity alterations.

5 Conclusions

The current experiment is considered a novel study that explains the molecular and biochemical mechanisms for the acclimation to hypersalinity stress in common carp. Total serum protein, albumin, and globulins decreased significantly, while blood glucose, circulating cortisol, Na^+, K^+, and Cl^- increased, by the

adverse effect of hypersalinity. In addition, hypersalinity is considered an immune and stress stimulant by upregulating gene expression of pro-inflammatory cytokines, $TNF-\alpha$, and $IL-1\beta$, as well as CRH genes in the liver, kidney, and spleen. Moreover, the acclimation of common carp to hypersalinity is represented by a marked upregulation of CAT gene expression in the liver without changes in the kidney and spleen. It is worth mentioning that the salinity challenge had a minimum effect on the gill tissue by regulating the $IL-1\beta$ gene only without changes in $TNF-\alpha$, CRH, and CAT gene expression. Furthermore, future study is required, using advanced techniques such as electron microscopy for an in-depth clarification of the activity and number of chloride cells inside the gill's microstructure as the main osmoregulatory organ in hypersalinity conditions. In addition, further molecular study is necessary for Na^+/K^+ $ATPase$ and Aquaporin gene expression which are the main contributors of homeostasis to hypersalinity.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

The animal study was reviewed and approved by the Ethical Research Committee of Faculty of Veterinary Medicine in South Valley University, Egypt under approval number (No. 48/12.9.2022).

Author contributions

HA, KB, and WE: Conceptualization, Formal analysis, Methodology, Investigation, Writing—original draft, and Writing – review and editing. AA, MA, and MS: Conceptualization, Formal analysis, Methodology, Data curation, Formal analysis, Writing—original draft, and Writing – review and editing. HE, ME, and SB: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Validation, and Writing—original draft. LF and SI: Resources, Software, Data curation, Validation, Visualization, Writing – original draft, Writing – review and editing, and Funding acquisition. FI: Software, Data curation, Validation, Writing –

review and editing, and Funding acquisition. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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