

Investigation of TNF- α and SAA Levels in the Pathogenesis of Time-Related Inflammatory Reaction in Zebrafish (*Danio rerio*)

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How to Cite

Ulucan, A., Yakut, S., Yüksel, H. (2026). Investigation of TNF- α and SAA Levels in the Pathogenesis of Time-Related Inflammatory Reaction in Zebrafish (*Danio rerio*). *Turkish Journal of Fisheries and Aquatic Sciences*, 26(7), TRJFAS27693. <https://doi.org/10.4194/TRJFAS27693>

Article History

Received 08 January 2025

Accepted 21 November 2025

First Online 12 February 2026

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Keywords

Immunohistochemistry

Inflammation

SAA

Spleen

TNF- α

Abstract

Zebrafish (*Danio rerio*) are valuable model organisms for studying inflammation. This study aimed to evaluate the role of TNF- α and SAA in time-dependent inflammatory responses using histopathological, immunohistochemical, and ELISA techniques. One-year-old wild-type zebrafish were divided into five groups (n=20). Group 1 (G-1) received intraperitoneal (IP) injections of 0.9% NaCl, while Groups 2-5 were treated with Complete Freund's Adjuvant and Keyhole Limpet Hemocyanin. G-2 and G-3 received a single dose on day 0 to model peracute and acute inflammation, while G-4 and G-5 received two doses on days 0 and 14 to model subacute and chronic inflammation. Blood and spleen samples were collected for further analysis.

The highest TNF- α and SAA immune-positivity intensity (IPI) scores were observed in G-4 and G-5, with staining in the perifollicular macrophages and lymphocytes. Plasma TNF- α peaked in G-4 (21.435 \pm 3.783 ng/L), and SAA in G-5 (1.657 \pm 0.218 μ g/ml). Significant correlations between TNF- α and SAA levels were found in G-2 (P<0.05). TNF- α and SAA levels showed statistical significance across different groups. These findings suggest that inflammatory cell accumulation via TNF- α and SAA plays a key role in zebrafish inflammation, providing insights into the molecular pathogenesis of time-dependent inflammatory responses.

Introduction

Inflammation, triggered by harmful stimuli like pathogens or damaged cells, activates immune and nonimmune cells to remove harmful factors, promote healing, and facilitate the interaction between innate and adaptive immune cells (Mathias et al., 2006). Cytokines are released, leading to symptoms like pain, redness, and swelling (Iribarne, 2021; Netea et al., 2017). While inflammation is essential for defense, excessive or prolonged inflammation can worsen disease and contribute to inflammatory disorders (Netea et al., 2017). Inflammation primarily functions as a beneficial defense against infection. However, acute or chronic excessive activation of the inflammatory reaction is also known to exacerbate the pathology of infectious diseases. The effects of inflammation extend

beyond the inflamed site, exerting systemic influences on various physiological processes (Campos-Sánchez and Esteban, 2021). Given the multifaceted nature of inflammatory responses, using experimental model organisms has become essential to dissect underlying cellular and molecular mechanisms. In this context, the zebrafish (*Danio rerio*) has emerged as a powerful model due to its unique biological and genetic advantages for investigating inflammation. In particular, when the unique properties of the zebrafish are studied in vivo and in real-time, novel host-pathogen relationships are revealed and immune mechanisms are uncovered (Mathias et al., 2006). It is also known to be a useful model organism for studying inflammatory regulation in human disease (Campos-Sánchez and Esteban, 2021). Many different transgenic strains of zebrafish allow the live imaging of innate and adaptive immune cells, which

include neutrophils (He et al., 2022; Mathias et al., 2006; Murdoch et al., 2019), macrophages (Iribarne, 2021), and lymphocytes (Hui et al., 2017; Iribarne, 2021).

Pro-inflammatory cytokines, such as TNF- α , serve as potential biomarkers for inflammatory diseases, aiding in their diagnosis and treatment (Iribarne, 2021; Siddhu et al., 2022). Inflammation activates various metabolic pathways and signaling molecules (He et al., 2022). TNF- α , primarily produced by activated macrophages and lymphocytes, plays a key role in immune responses and can induce apoptotic cell death, contributing to organ damage (Churchill et al., 2020). Lipopolysaccharide (LPS), a potent macrophage activator, induces pro-inflammatory molecules, including cytokines like TNF- α , as part of the inflammatory response (Zhang et al., 2019).

Serum amyloid A (SAA) is an acute-phase protein that increases during inflammation (Kovacevic and Belosevic, 2015; Villarroel et al., 2008). It aids in zebrafish development (Joshi et al., 2009) and is upregulated by TNF- α (Iribarne, 2021). SAA has cytokine-like properties, attracting immune cells like phagocytes and neutrophils (He et al., 2009; Murdoch et al., 2019). Found in various fish species, SAA plays a role in immune modulation (Kovacevic and Belosevic, 2015).

This study aimed to investigate the relationship between TNF- α and SAA levels in the pathogenesis of time-dependent inflammatory reactions in zebrafish using histopathological, immunohistochemical, and ELISA techniques. Thus, we discuss the mechanisms of action, roles, and pathogenesis of TNF- α , a pro-inflammatory cytokine, and SAA, an important acute phase protein, in different inflammatory processes of peracute, acute, subacute, and chronic types in blood plasma and spleen tissue, which have important roles in immune system functions in zebrafish.

Materials and Methods

Animals

In this study, 100 wild-type (AB strain) zebrafish aged 1 year were used. The experiment was conducted at Bingöl University Laboratory Animal Research Center, with a light intensity of 40 lux and a water temperature of 28.5 °C. The fish were divided into five groups (n=20) in separate 40-liter aquariums and fed twice daily with commercial tropical fish flakes, with excess food removed after feeding (Nusslein-Volhard and Dahm, 2002).

Preparation and Application of Complete Freund's Adjuvant and Keyhole Limpet Hemocyanin Emulsion

The emulsion was prepared by mixing (1 ml/1 mg ratio) 1 mg of Keyhole Limpet Hemocyanin (KLH) (374805, Sigma, USA) in each 1 ml of Complete Freund's Adjuvant (CFA) (F5881-10X, Sigma, USA). CFA-KLH emulsion was injected intraperitoneally (IP) from the

midline of the pelvic fins with the help of a 35 μ l microinjector at a dose of 5 μ l to each fish to which the inflammatory reaction was to be induced (Kinkel et al., 2010; Lugo-Villarino et al., 2010; Page et al., 2013).

Experimental Study Design

In the study, G-1 (Control) fish were injected with 5 μ l sterile 0.9% NaCl on days 0 and 14, and samples were collected on day 21. In other groups, inflammatory reactions were induced as follows: G-2 (P-act, Peracute) received a 5 μ l CFA-KLH emulsion injection on day 0, with samples collected on day 1; G-3 (Act, Acute) received the same injection on day 0, with samples taken on day 7; G-4 (S-Act, Subacute) received the injection on days 0 and 14, with samples collected on day 14; G-5 (Chr, Chronic) received the injection on days 0 and 14, with samples collected on day 21 (Lugo-Villarino et al., 2010; Page et al., 2013).

Anesthesia, Necropsy, Processing of Blood and Tissue Samples

Fish were anesthetized with tricaine methanesulfonate (TMS) (Tricaine-S[®]) (Western Chemical Inc., Ferndale, USA) at a dose of 0.168 mg/ml. Blood samples were collected from the dorsal aorta using a microhematocrit tube (088.02.001, Interlab, Türkiye) after tail amputation under anesthesia. The samples were then centrifuged at 3000 rpm for 10 minutes using a microhematocrit centrifuge (Hematocrit 210, Hettich, Germany) to separate the plasma for serological analysis. The tissues of the euthanized and necropsied fish were taken as a whole block. The tissues of all fish were fixed in 10% buffered formaldehyde for 48 h and embedded and solidified into blocks in paraffin after routine histopathological procedures. Paraffin-embedded tissues were cut into 4 μ m thickness with a rotary microtome (RM 2155, Leica, Germany) and transferred to the adhesive slides. Slides were stained with Hematoxylin and Eosin (H&E) for routine histopathological examination and analyzed for histopathologic changes in spleen tissue sections and photographed with an imaging system adapted light microscope (ECLIPSE 80i, C-SP 756526, Nikon, Japan).

Histopathological Examinations

Histopathological staining and examinations were applied to the spleen tissues of all 20 fish in each group. Leukocyte infiltration frequency (LIF) (%) scores of spleen tissues related to inflammatory reactions were examined microscopically at 20x objective magnification in five randomly chosen, non-overlapping fields and evaluated semi-quantitatively according to a small modification of the literature data. Accordingly, LIF scores were classified as follows: none (0); poor, few lymphoid areas (less than 1%=1); mild, some lymphoid areas (1-9%=2); moderate, some or all lymphoid areas

(10-32%=3); moderate/significant, all lymphoid areas (33-65%=4); and significant, all lymphoid areas (>65%=5) (Belo et al., 2021).

Immunohistochemical Examinations

Spleen tissue sections were deparaffinized with xylene, rehydrated with graded alcohol, and stained using a modified SABC-P method. Endogenous peroxidase activity was blocked with 3% H₂O₂, followed by antigen retrieval in citrate buffer (pH 6.0) using a microwave. Immune reactivity was detected with anti-TNF- α and anti-SAA antibodies. Protein blocking, secondary antibody, and enzyme complexes were applied according to the conjugation kit protocol. The color reaction was developed using DAB, and background staining was performed using Mayer's hematoxylin. Slides were washed, dehydrated, cleared, and mounted, with controls used. IHC staining and evaluation were performed on the spleen tissues of all 20 fish in each group. Five randomly selected areas in the stained spleen sections from each group were examined under a 20x objective magnification using a semi-quantitative method. The immune-positivity intensity (IPI) was scored as follows: no positivity (negative, 0), light-brown particle (weak, +1), brown particle (moderate, +2), and dark brown particle (severe, +3) (Belo et al., 2021).

Serological Examinations

SAA and TNF- α levels were determined by the Sandwich ELISA method using ELISA kits (YLA0072FI, and YLA0053FI, ELISA kit, China). ELISA kit protocols were administered according to the manufacturer's directions when preparing samples and reagents. Standard solutions and detection curves for each administration were previously made available in the ELISA reader device (SpectraMax Plus 384, USA). The plasma samples' optical density (OD) values were automatically measured at 450 nanometers (nm) using an ELISA reader (Ozgocer et al., 2017).

Statistical Evaluation

Statistical analysis was performed using SPSS 18.0.0 for Windows to evaluate the distribution of inflammatory reaction scores in the tissues and ELISA results. Differences between groups were assessed using "Tukey", "ANOVA", and "Kruskal-Wallis" tests. The Chi-square test was applied to the inflammatory reaction scores. Pearson Correlation was used to assess relationships between plasma values of each experimental group, with correlation coefficients' significance checked by the "t-test". P<0.05 was considered statistically significant, and P<0.01 was considered statistically very significant.

Results

Clinical and Macroscopic Results

After the experimental procedure, no clinical or macroscopic changes were observed in the control group. In the inflammation-induced groups, a temporary reduction in physical activity was observed for approximately 2 hours following CFA-KLH injection. Specifically, the zebrafish swam less frequently and at a slower pace, with many fish remaining close to the water surface. There was also reduced responsiveness to external stimuli, and the injection site showed mild erythema.

Necropsy revealed adhesions between the visceral organs and peritoneum (intraperitoneal fibrosis) in the subacute (G-4/S-act) and chronic inflammation groups (G-5/Chr) (Figure 1).

Histopathologic Results

Spleen tissue inflammation, assessed using LIF scores (Table 1), showed higher inflammation in G-3 (Act), G-4 (S-Act), and G-5 (Chr). The G-1 (Control) group exhibited normal histologic architecture (LIF score = 0). In contrast, CFA-KLH-treated groups revealed the presence of neutrophils, lymphocytes, and macrophages in different stages of inflammatory response. G-2 (P-Act) showed moderate inflammation

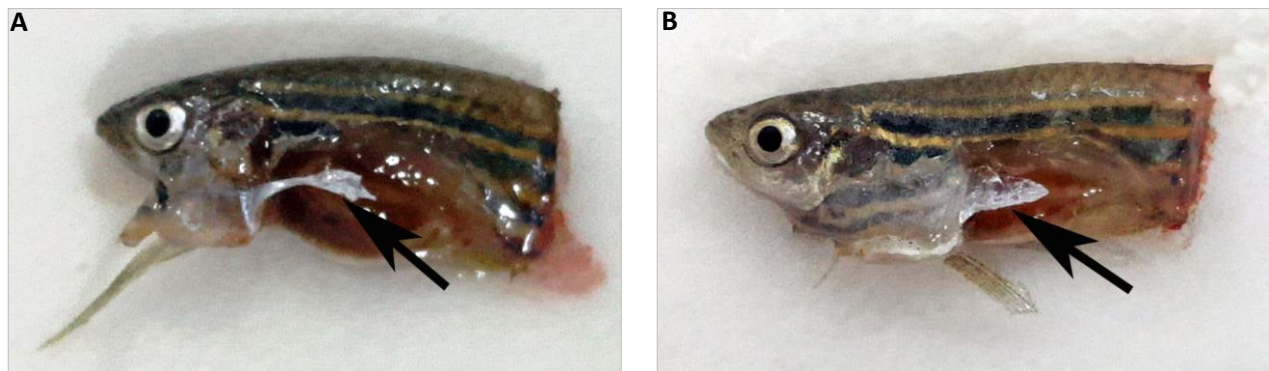


Figure 1. Macroscopic view of the necropsied zebrafish. (A) Adhesions (intraperitoneal fibrosis) between visceral organs and peritoneum in the abdominal cavity in the subacute inflammation group (G-4/S-Act); (B) adhesions between visceral organs and peritoneum in the chronic inflammation group (G-5/Chr).

(+3). G-3 (Act) demonstrated marked inflammation (+5), G-4 (S-Act) displayed moderate to marked changes (+4), and G-5 (Chr) exhibited the most severe lesions (+5). G-3 (Act) showed necrotized foci, whereas G-4 (S-Act) and G-5 (Chr) had lymphoid depletion and follicle enlargement. Although the inflammatory severity in G-4 (S-Act) was slightly lower than in G-3 (Act) and G-5 (Chr), histologic alterations such as cellular shrinkage, increased intercellular space, and partial lymphoid depletion were evident. These findings indicate progressive tissue damage, reaching its maximum extent in G-5 (Chr) (Figure 2,3).

Immunohistochemical Results

IHC staining of spleen tissues with anti-TNF- α and anti-SAA antibodies was evaluated using IPI scores (Table 2). A direct correlation was found between G-1 (Control), G-2 (P-Act), and G-3 (Act), and an inverse correlation was found between G-4 (S-Act) and G-5 (Chr) for TNF- α and SAA. In the control group, no staining was observed (IPI score 0). The highest TNF- α IPI score (+3) was seen in G-4 (S-Act), and the highest SAA IPI score (+3) was in G-5 (Chr). Immuno-positive staining was observed in macrophages and lymphocytes in the perifollicular areas of the spleen, corresponding with histopathological lesions and LIF scores (Figure 3,4).

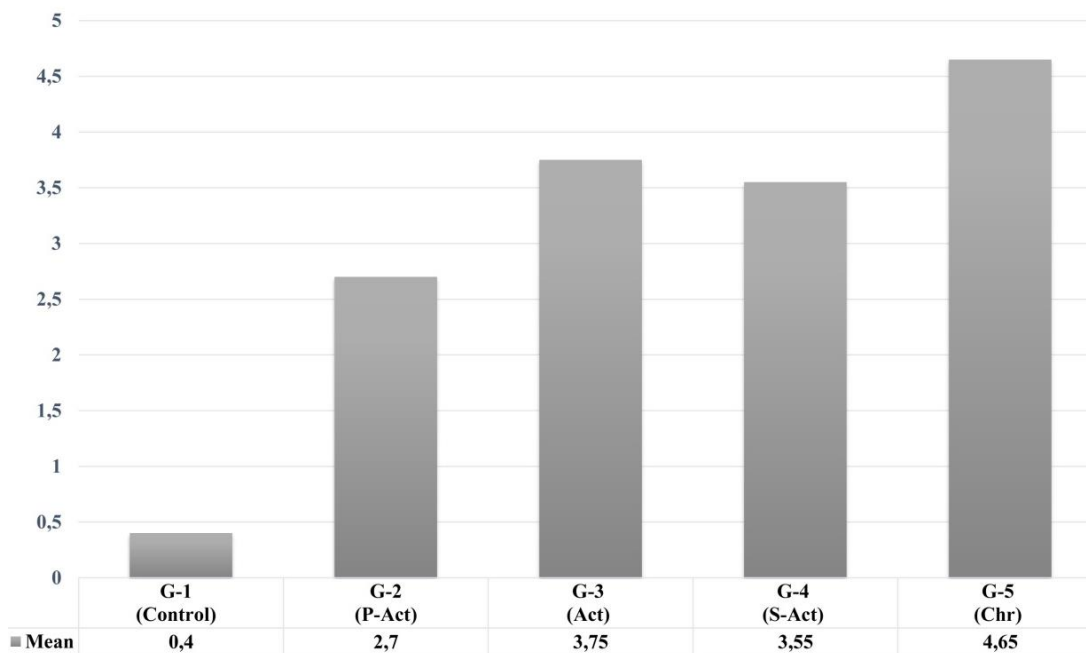
ELISA Results

Table 1. Percentages and values of histopathologic LIF (%) scores* in spleen tissues of the experimental groups

Markers	Groups	None (0)	Weak (1)	Moderate (2)	Severe (3)	Total No/%
TNF- α	G-1 (Control)	No/%	12/60%	8/40%	0/0%	20/100%
	G-2 (P-Act)	No/%	0/0%	16/80%	4/20%	20/100%
	G-3 (Act)	No/%	0/0%	3/15%	12/60%	20/100%
	G-4 (S-Act)	No/%	0/0%	3/15%	8/40%	20/100%
	G-5 (Chr)	No/%	0/0%	6/30%	14/70%	20/100%
Total	No/%	12/12%	36/36%	38/38%	14/14%	100/100%
SAA	G-1 (Control)	No/%	16/80%	4/20%	0/0%	20/100%
	G-2 (P-Act)	No/%	0/0%	20/100%	0/20%	20/100%
	G-3 (Act)	No/%	0/0%	12/60%	8/40%	20/100%
	G-4 (S-Act)	No/%	0/0%	14/70%	6/30%	20/100%
	G-5 (Chr)	No/%	0/0%	0/0%	9/45%	11/55%
Total	No/%	16/16%	50/50%	23/23%	11/11%	30/100%

*Distribution of differences in LIF scores between groups according to experimental groups by percentile using the Pearson chi-square test.

Mean values of histopathologic LIF (%) scores of the experimental groups



LIF (%) score classification: None (0); Weak (1); Mild (2); Moderate (3); Moderate/marked (4); Marked (5)

Figure 2. Mean values of histopathologic LIF (%) scores of the experimental groups. The mean histopathologic LIF (%) scores of the spleen tissues of the fish in the experimental groups were as follows: G-5 (Chr), G-3 (Act), G-4 (S-Act), G-2 (P-Act), and G-1 (Control).

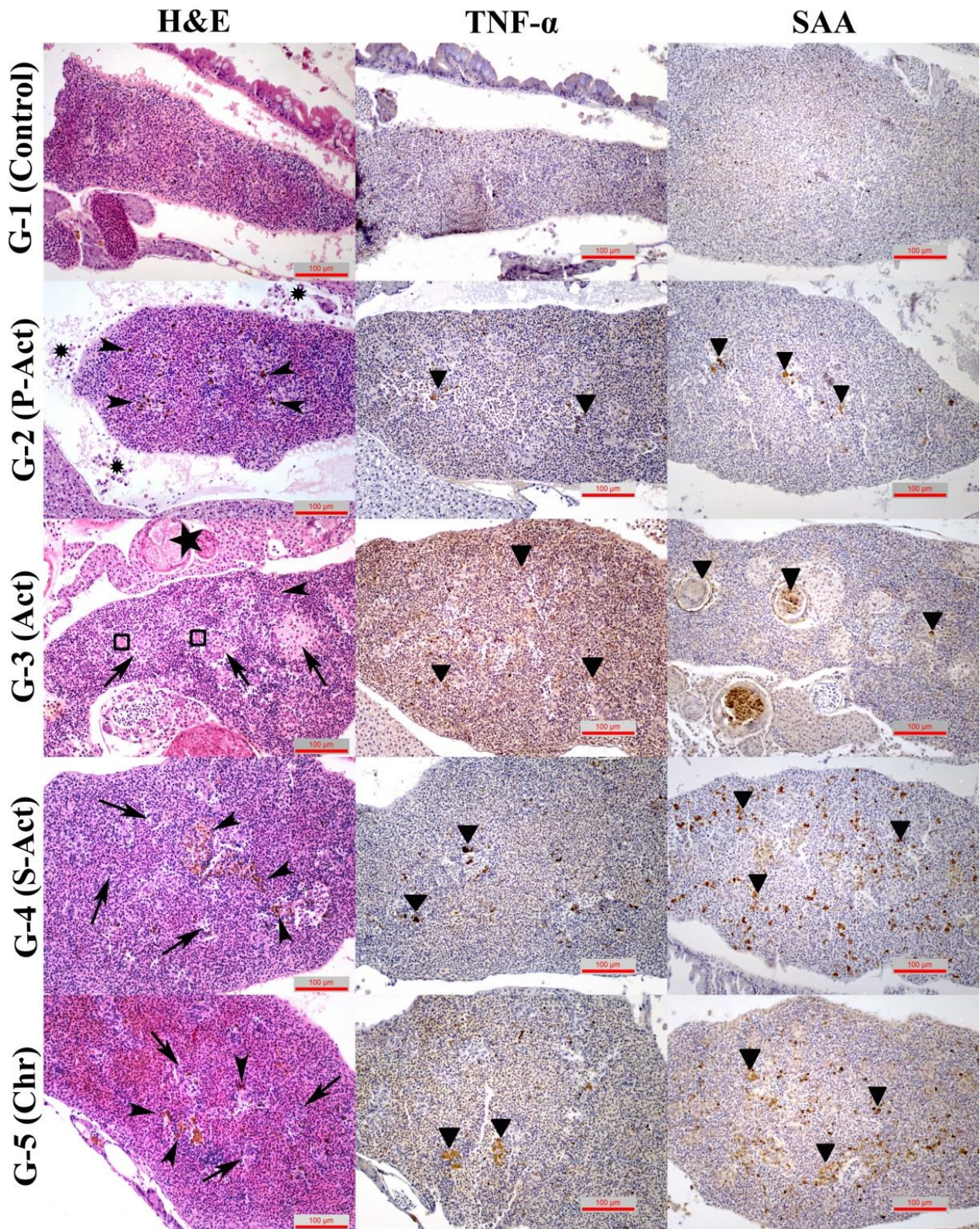


Figure 3. Microscopic view of H&E and IHC-stained spleen tissues of zebrafish. Column H&E: G-1 (Control) normal histological appearance of spleen tissue (LIF score=0). G-2 (P-Act) moderate (+3), G-3 (Act) marked (+5), G-4 (S-Act) moderate/marked (+4), and G-5 (Chr) marked (+5) histopathologic appearance of LIF scores. Increased number of melanoma-macrophages (arrowheads); enlargement of lymphoid follicles and lymphoid depletion (arrows); accumulation of hyalineous material (square frame); abscesses (five-pointed star). H&E staining, x200 magnification. Columns Anti-TNF- α and Anti-SAA: Presence of immune-reactivity in spleen tissues of experimental groups. Triangles indicate immunopositively stained areas. The IPI scores of TNF- α were as follows: G-1 (Control), negative (0); G-2 (P-Act), weak (+1); G-3 (Act), severe (+3); G-4 (S-Act), moderate (+2); and G-5 (Chr), severe (+3). The IPI scores of SAA were as follows: G-1 (Control), negative (0); G-2 (P-Act), weak (+1); G-3 (Act), moderate (+2); G-4 (S-Act), severe (+3); and G-5 (Chr), severe (+3). Immunohistochemical SABC-P staining method, x200 magnification.

TNF- α and SAA levels were measured in plasma (ng/L and $\mu\text{g/ml}$, respectively) using ELISA. The group averages for plasma TNF- α and SAA levels are shown in Figure 5A and 5B, respectively. A significant correlation ($P<0.05$) between TNF- α and SAA levels was found in G-2 (P-Act) (Figure 5C). The highest TNF- α level was in G-4 (S-Act) (21.435 ± 3.783 ng/L) and the highest SAA level was in G-5 (Chr) (1.657 ± 0.218 $\mu\text{g/ml}$). Statistical analysis revealed significant differences in TNF- α levels between G-2 (P-Act) and G-4 (S-Act), and G-3 (Act) and G-4 (S-Act) ($P<0.01$) (Figure 6A). For SAA, a significant difference between G-2 (P-Act) and G-5 (Chr) was found ($P<0.05$) (Figure 6B).

Discussion

Inflammation defends against pathogens and injury, aiding tissue healing and homeostasis. However, if uncontrolled, it can cause tissue damage, and metaplasia, and disrupt balance (Campos-Sánchez and Esteban, 2021; Hwang et al., 2016; Medzhitov, 2008). Immune cells and tissue-resident cells regulate inflammation using various molecules and mechanisms (Belo et al., 2021; Campos-Sánchez and Esteban, 2021). We created a time-sequential zebrafish model of inflammation (peracute to chronic) to study TNF- α , SAA, and inflammatory cellular reactions. This pioneering model provides insights into the pathogenesis of inflammation at all stages. Zebrafish, with their conserved nociceptive mechanisms, are used in

Table 2. Percentages and values of IHC IPI scores* in spleen tissues of the experimental groups

Groups	None (0)	Weak (1)	Mild (2)	Moderate (3)	Moderate/Marked (4)	Marked (5)	Total No/%
G-1 (Control)	No/0%	12/60,0%	8/40,0%	0/0,0%	0/0,0%	0/0,0%	20/100%
G-2 (P-Act)	No/0%	0/0,0%	6/30,0%	14/70,0%	0/0,0%	0/0,0%	20/100%
G-3 (Act)	No/0%	0/0,0%	1/5,0%	10/50,0%	2/10,0%	7/35,0%	20/100%
G-4 (S-Act)	No/0%	0/0,0%	0/0,0%	9/45,0%	11/55,0%	0/0,0%	20/100%
G-5 (Chr)	No/0%	0/0,0%	0/0,0%	0/0,0%	7/35,0%	13/65,0%	20/100%
Total	No/0%	12/12%	8/8%	7/7%	33/33%	19/19%	100/100%

*Distribution of differences in IPI scores between groups according to experimental groups by percentile using Pearson's chi-square test.

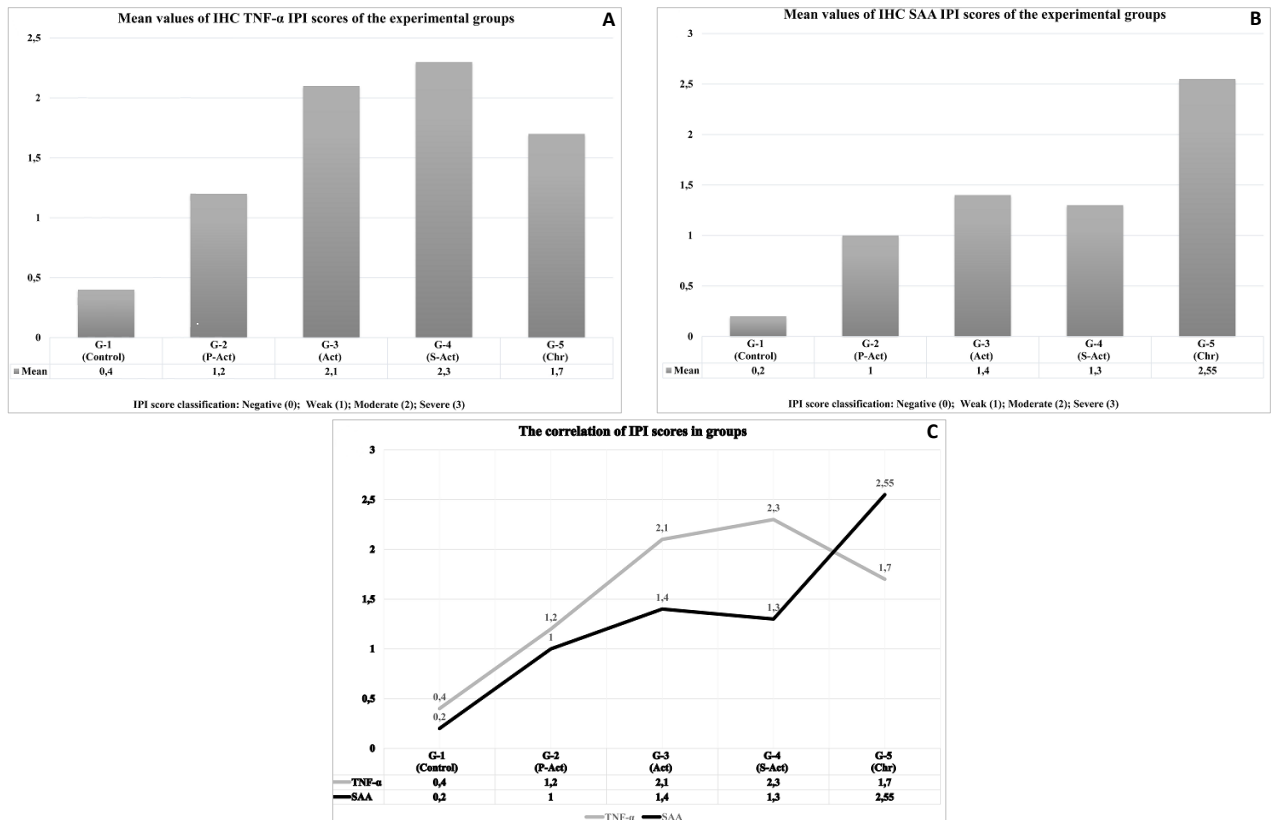


Figure 4. Mean values of IHC IPI scores and correlations in the experimental groups. (A) Mean values of TNF- α IPI scores of the experimental groups. The mean IHC TNF- α IPI scores of the spleen tissues of the fish in the experimental groups from high to low, respectively: G-4 (S-Act), G-3 (Act), G-5 (Chr), G-2 (P-Act), G-1 (Control). (B) Mean SAA IPI scores in the experimental groups. The mean IHC SAA IPI scores of the spleen tissues of the fish in the experimental groups were ranked from high to low as follows: G-5 (Chr), G-3 (Act), G-4 (S-Act), G-2 (P-Act), and G-1 (Control). (C) IPI score correlation of IHC TNF- α and SAA findings between groups. Presence of a directly proportional correlation between G-1 (Control), G-2 (P-Act), and G-3 (Act) and an inverse proportional correlation between G-4 (S-Act) and G-5 (Chr).

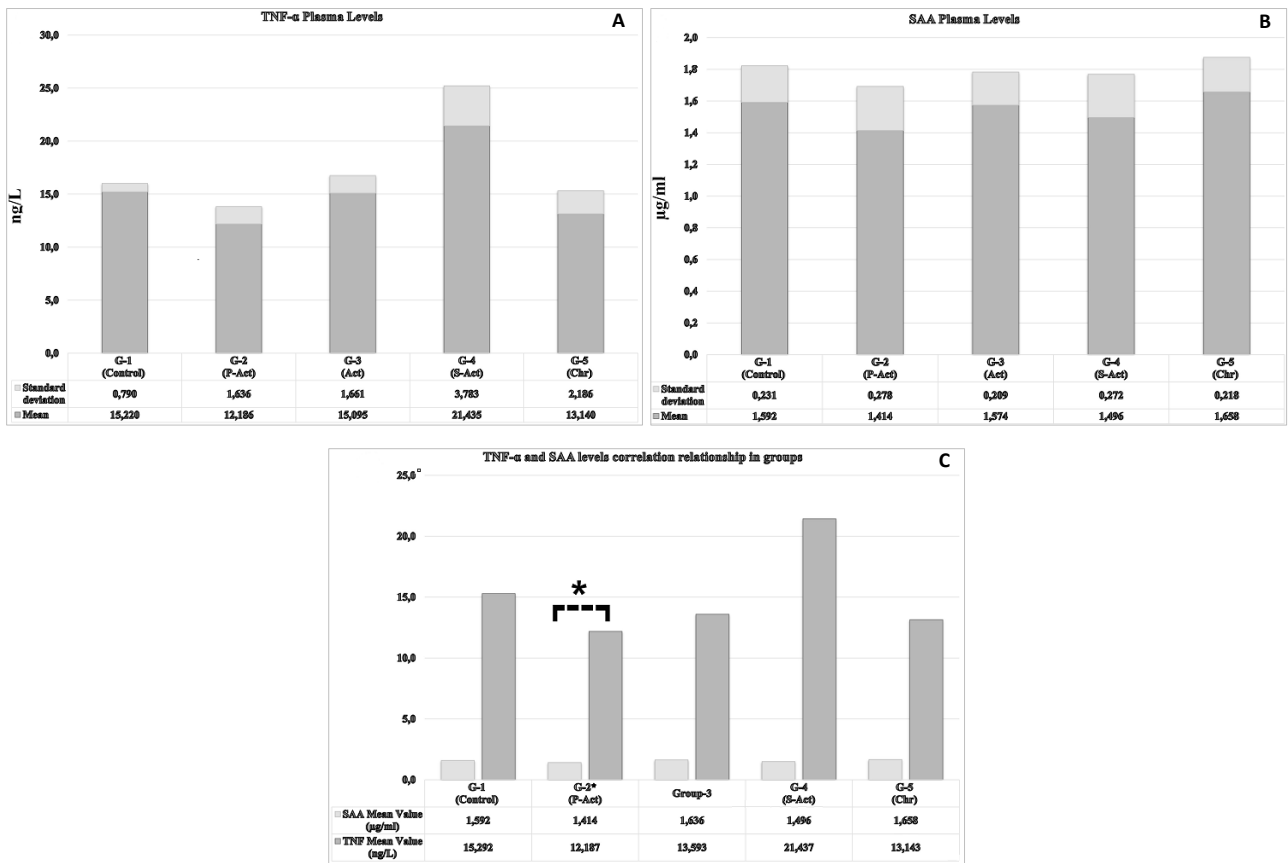


Figure 5. Mean values of ELISA levels and correlations in the experimental groups. (A) Mean plasma TNF-α levels (ng/L). (B) Mean plasma SAA values (µg/ml). (C) Intergroup correlation between TNF-α and SAA levels (Pearson's correlation test). *According to the correlation data between TNF-α and SAA levels, a significant level correlation (P<0.05) was found in G-2 (P-Act).

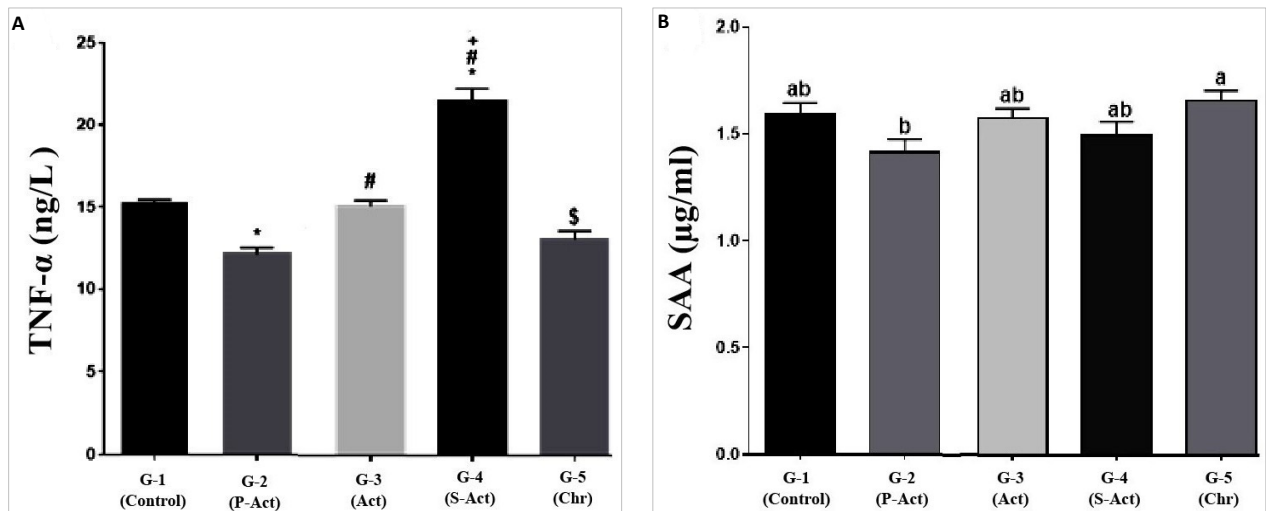


Figure 6. Statistical differences in ELISA levels according to the experimental groups. (A) Statistical differences in plasma TNF-α levels. The statistical difference between G-2 (P-Act) and G-4 (S-Act) and between G-3 (Act) and G-4 (S-Act) is highly significant (P<0.01). Differences between groups, indicated by different signs in the graph columns, were statistically significant (P<0.01) (Kruskal-Wallis test for comparison of different groups of data). (*) indicates a significant difference from G-1 (Control), (#) indicates a significant difference from G-2 (P-Act), (+) indicates a significant difference from G-3 (Act), and (\$) indicates a significant difference from G-4 (S-Act). (B) Statistical differences in plasma SAA levels. The statistical difference between G-2 (P-Act) and G-5 (Chr) is significant (P<0.05). (a-b): Differences between groups indicated by different letters in the graph columns are statistically significant (P<0.05) (Tukey Post Hoc., Comparison of Different Data Groups Test).

inflammation studies. Larvae show increased movement in response to painful stimuli like temperature or chemicals (Campos-Sánchez and Esteban, 2021). While zebrafish studies on inflammation and related proteins are common, data on inflammatory pathogenesis across different phases are limited. Our study examined spleen tissue for TNF- α and SAA, offering insights into inflammation mechanisms at various stages.

CFA, containing LPS from heat-killed *Mycobacterium tuberculosis*, is used in fish to recruit macrophages and other inflammatory cells to the injection site, enhancing the immune response (Ina-Salwany et al., 2023). LPS treatment can cause oxidative stress, cell death, and lipid peroxidation (Campos-Sánchez and Esteban, 2021; Siddhu et al., 2022). Pretreating zebrafish with low non-lethal doses of LPS followed by high lethal doses prevents death, suggesting that immune tolerance observed in mammals can be replicated in zebrafish (Campos-Sánchez and Esteban, 2021; Xie et al., 2021). Also, a decrease in the survival rate of zebrafish larvae was reported in the in-vivo zebrafish larvae toxicity assay induced with LPS (0.5 $\mu\text{g}/\text{mL}$) (Siddhu et al., 2022). Our study revealed that giving zebrafish the CFA-KLH emulsion through the peritoneum caused both macroscopic and microscopic inflammatory changes. This was shown by histopathology, immunohistochemistry, and plasma TNF- α and SAA levels, which showed that it worked to cause inflammation.

Zebrafish inflammation models show morphological damage in the spleen and other organs, with significant pathological changes in morphometric evaluations (Belo et al., 2021). Zebrafish subjected to experimentally induced inflammation demonstrated distinct histopathological changes in the spleen, including cellular atrophy, enlarged intercellular spaces, mitochondrial edema, elevated erythrocyte density, vascular congestion, and perivascular hemorrhages (Chen et al., 2016). Neutrophils, as initial leukocytes responding to tissue damage, signify the shift from acute to chronic inflammation as their quantity escalates (Belo et al., 2021; Iribarne, 2021). Likewise, zebrafish subjected to a 3-hour hypoxic injury exhibited a notable increase in neutrophil migration relative to controls (He et al., 2022). The overall histopathological pattern observed in our study closely corresponds with the recognized inflammatory progression delineated in zebrafish models. In accordance with the findings of Belo et al. (2021) and Chen et al. (2016), the noted cellular degeneration and vascular alterations indicate ongoing tissue damage during the acute phase. The predominance of neutrophils in the peracute and acute stages, succeeded by progressive infiltration of lymphocytes and macrophages in the subacute and chronic phases, reflects the sequential activation of TNF- α and SAA observed in our data. Increased TNF- α expression occurred alongside neutrophilic infiltration, while SAA immunoreactivity correlated with macrophage-dominant phases, underscoring the

progression from acute to chronic inflammation and the initiation of tissue remodeling.

Pro-inflammatory cytokines like TNF- α are used to assess inflammation (Iribarne, 2021). In zebrafish larvae treated with LPS, TNF- α expression increased (Siddhu et al., 2022; Zhang et al., 2019). Studies showed significant up-regulation of TNF- α mRNA 3 hours after LPS injection (He et al., 2022) with peaks at 6 hours in larvae exposed to LPS (Philip et al., 2014). While peak expression occurs within 3-6 hours, its duration is unclear. Thus, evaluating only plasma TNF- α may not fully reflect differences between control and inflammatory groups. The higher TNF- α levels in the subacute phase could be due to a new inflammatory stimulus during acute inflammation.

In chronic inflammation, TNF- α is released from the local site (Campos-Sánchez and Esteban, 2021; Iribarne, 2021), guiding leukocyte migration (Belo et al., 2021; Iribarne, 2021; Kyritsis et al., 2012). TNF- α is also a key signal released by polarized macrophages in early regeneration stages (Iribarne, 2021; Nguyen-Chi et al., 2017). The highest IHC TNF- α IPI score in the spleen, reflecting subacute inflammation, aligns with ELISA findings. Anti-TNF- α staining in macrophages and lymphocytes correlates with histopathological lesions and LIF scores, linking TNF- α with increased leukocyte infiltration in zebrafish spleen tissue.

An increase in the immune-related genes TNF- α with transcriptional changes was reported in the spleen of zebrafish after inflammatory stimulation (Chen et al., 2016). The light-dark cycle has been reported to have a major effect on immunity in zebrafish, similar to that in mammals. In zebrafish larvae infected with *Salmonella Typhimurium* or exposed to constant light during the light phase of a light-dark cycle, increased neutrophil and macrophage infiltration into infection sites and increased TNF α expression were demonstrated (Du et al., 2017; Iribarne, 2021). In zebrafish, TNF- α levels in spleen tissue and plasma can increase or decrease during the time-dependent inflammatory reaction process. Increased TNF- α levels are in parallel with the severity of the inflammatory reaction and histopathological lesions. Therefore, an increase in TNF- α levels in zebrafish accompanies increased cellular damage and inflammatory response.

SAA was first reported as an acute phase protein in trout. Hepatocytes of salmonids have been reported to be an important source of SAA. However, SAA synthesis in hepatocytes of other fish species is not well known (Jørgensen et al., 2000). Acute phase proteins are currently used as possible biomarkers for monitoring animal welfare and health situation and SAA is one of them. SAA expression was primarily detected in the tissues of the liver, gill, muscle, skin, and head kidney in orange-spotted grouper (*Epinephelus coioides*) (Wei et al., 2013). In many teleost infection models, upregulation of liver SAA expression suggests that SAA acts as a positive acute phase protein (Castellano et al., 2020). Although SAA is present in some tissues,

especially liver tissue, in fish, data on the detection of SAA in spleen tissue in zebrafish are very limited. In our study, we revealed the presence of SAA as IHC in the time-related inflammatory reaction model as new data in the spleen tissue of zebrafish.

SAA is a key acute-phase reagent in vertebrates, with fish SAAs differing from mammals. Goldfish SAA is closely related to other cypriniform species (Kovacevic and Belosevic, 2015). In rainbow trout, SAA secretion was linked to immune responses, but plasma levels did not significantly increase (Villarroel et al., 2008). In our study, immuno-positive SAA staining in zebrafish macrophages was observed, but no significant plasma SAA difference was found between healthy and tissue-damaged fish, suggesting another acute-phase protein may be a more active inflammatory biomarker.

SAA levels can increase up to 1000-fold during inflammation, remaining elevated for 7 to 11 days before returning to normal. While hepatic SAA response has been studied, its correlation with serum levels is rarely examined due to detection challenges (Castellano et al., 2020). Our study is the first to examine acute phase response factors in zebrafish across various inflammatory phases in both tissues and plasma. Using the IP CFA-KLH injection, we observed significant acute phase responses through histopathological, immunohistochemical, and serological measurements of SAA and TNF- α .

In zebrafish, SAA plays roles in cell chemotaxis, acute inflammatory response, stress, injury, bacteria response, and locomotor behavior (Castellano et al., 2020; Gutha et al., 2018). SAA's presence and structure are similar to mammals, with a significant 500-fold increase in levels after bacterial infections, comparable to humans and mice (Lin et al., 2007). In sturgeons, hepatic SAA expression after bacterial stimulation peaks on day 3 and persists for weeks, although it is weaker than in mammals (Castellano et al., 2020). SAA expression is absent in germ-free zebrafish larvae but induced after microbial colonization (Murdoch et al., 2019). Plasma SAA levels in zebrafish differ significantly between peracute and chronic inflammation ($P < 0.05$). Unlike mammals, where SAA levels rise rapidly during inflammation, zebrafish exhibit a slower increase in time-dependent inflammatory reactions.

In zebrafish, anti-SAA staining in macrophages and lymphocytes in spleen perifollicular areas correlates with histopathological lesion severity. As inflammation progresses from acute to chronic in zebrafish, tissue SAA levels rise, while plasma levels fluctuate.

In goldfish, like mammals, SAA is highly expressed in the kidney, spleen, and intestines, with the highest SAA mRNA levels found in neutrophils and the lowest in macrophages (Castellano et al., 2020; Kovacevic and Belosevic, 2015). SAA has also been observed in rainbow trout macrophages treated with LPS (Goetz et al., 2004). *Trypanosoma carassii* infection in goldfish caused sustained up-regulation of SAA in the kidney, liver, and spleen. In goldfish monocytes, recombinant SAA protein

increased TNF- α expression at various time points (Kovacevic and Belosevic, 2015). In zebrafish, anti-SAA staining in macrophages and lymphocytes in spleen perifollicular areas correlates with histopathological lesion severity. As inflammation progresses from acute to chronic in zebrafish, tissue SAA levels rise, while plasma levels fluctuate.

Conclusion

This study induced peracute, acute, subacute, and chronic inflammation in zebrafish using CFA-KLH emulsion, examining time-dependent inflammatory responses. We found that TNF- α and SAA levels increased in both spleen tissue and blood plasma. As inflammation progressed, TNF- α and SAA expression, along with neutrophil and macrophage infiltration, increased. Our findings show that zebrafish are an excellent model for studying inflammation across stages, helping to understand the accumulation of inflammatory cells and the roles of TNF- α and SAA in pathogenesis.

Ethical Statement

All procedures, treatments, and care applications on animals were carried out with the approval of Ethical Permit Number 09/02 dated 26.09.2014 obtained from Bingöl University Animal Experiments Local Ethics Committee.

Funding Information

This research was supported by the Scientific and Technological Research Council of Türkiye with project number 215Z467.

Author Contribution

First Author: Conceptualization, Writing -review and editing, Funding Acquisition, Project Administration, Resources, Writing -review and editing; Data Curation, Formal Analysis,

Second Author: Investigation, Methodology, Visualization and Writing -original draft;

Third Author: Supervision, Writing - review and editing

Conflict of Interest

The authors report no conflicts of interest.

Acknowledgements

We thank the relevant institution and all institution officials for this scientific research project (3001) supported by TUBITAK with project number 215Z467.

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