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Research Article

Total Sialic Acid, Oxidative Stress and Histopathological Changes in Rainbow Trout Saprolegniasis *(Oncorhynchus mykiss)*

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Abstract

Saprolegniasis is known as one of the most important fungal diseases of salmonids along with high mortality and economic problems. One hundred and seven fish suffering from cutaneous *Saprolegnia* infections and the same number of healthy fish were selected and blood parameters along with histopathology assay were performed in all ones. The results indicated a significant increase (P≤0.01) in total sialic acid, malondialdehyde, urea, creatinine, aspartate aminotransferase, total protein in plasma and a decrease in glucose, catalase, glutathione peroxidase and paraoxonase. Meanwhile, no significant alterations of alanine aminotransferase and superoxide dismutase were revealed in infected fish. Also, the histopathological findings observed in liver, especially glycogen storage and fatty inclusion and melanomacrophage centres in spleen. Tubular vascular degeneration along with cystic formation was identified in kidney. The results suggest that saprolegniasis develops substantial histopathological and blood profile changes in rainbow trout and recommend to pay more attention on some biochemical profiles such as MDA and TSA, due to cell health and defence against fungus on the skin respectively, along with hepatocyte function index (aspartate aminotransferase) and nitrogen metabolism (creatinine and urea) during disease management.

Keywords: *Saprolegniasis, Blood parameters, Histopathology, Rainbow trout*

Saprolegniyazis`li Gökkuşağı Alabalıklarında *(Oncorhynchus mykiss)*, Toplam Siyalik Asit, Oksidatif Stres ve Histopatolojik Değişiklikler

Özet

Saprolegniya enfeksiyonu salmonid balıklarda yüksek mortalite ve ekonomik sorunlarla birlikte en önemli mantar hastalıklardan biri olarak bilinir. Yüz yedi adet Saprolegniya enfekte ve aynı sayıda sağlıklı balıklar seçilerek, histopatolojik analizler`ile birlikte bazı kan parametreleri incelendi. Plazma toplam siyalik asit, malondialdehit, üre, kreatinin, aspartat aminotransferaz ve toplam protein düzeylerinde artış (P≤0.01) görülürken, glukoz miktarı miktarı`ile, katalaz, glutatiyon peroksidaz ve paraoksonaz aktivitesinde ise azalma (P≤0.01) görüldü. Alanin aminotransferaz ve süperoksit dismutaz aktivitesinde anlamlı bir değişiklik görülmedi. Ayrıca, karaciğerde gözlenen histopatolojik bulgularda, glikojen depolanması ve yağ içerme ve dalakta melanomakrofaj merkezleri görüldü. Böbrek`de kistik oluşum`ile birlikte tubular vacuolar dejenerasyonu tespit edildi. Sonuçlar gökkuşağı alabalığında önemli histopatolojik ve kan profil değişikliklerini sebep olduğunu gösteriyor ki bu arada MDA ve TSA tertible hücre sağlığı ve deride oluşan mantara karşı savunmada özel önem taşıyorlar. Ayrıca hepatosit fonksiyon indeksi (aspartat aminotransferaz) ve azot metabolizması (kreatinin ve üre) gibi bazı biyokimyasal parametrelerin hastalık yönetiminde önemli rolları olabiliceği düşünüldü.

Anahtar sözcükler: *Saprolegniasis, Kan parametreleri, Histopatoloji, Gökkuşağı alabalık*

INTRODUCTION

Fungal diseases involve severe management problems in aquaculture farms and saprolegniasis is known as one of the main types salmonids diseases [1,2]. Saprolegniasis in addition to salmonid fish, has been also reported in crayfish *(Astacus leptodactylus)* [3]. *Saprolegnia* as major genus of water molds of Oomycete class possesses opportunistic, saprotrophic and necrotrophic traits. Naturally, it is found in fresh water ecosystems and environmental factors such as low whirlpool, low soluble oxygen, stress and over-crowding facilitate saprolegniasis occurrence in aquaculture

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farms along with mortality in fresh water fish [4]. Acetylated derivative of neuraminic acid is sialic acid (SA) that is extensively dispensed in entirely vertebrate tissues, body fluids and in higher invertebrate [5,6]. It is worth mentioning that SA indwells the terminal location on macromolecules, cell membranes and it is known as an inflammatory marker [7-9]. Therefore, the determination of SA may be a valuable indicator for diagnosis and prognosis of inflammatory diseases. Also, evidences reveal the changes of serum SA in different diseases [5,10,11]. However, assay has not been observed about SA levels in fungal fish diseases such as saprolegniasis.

Free Radicals (FRs) such as superoxide anion, hydroxyl radical, as well as non-radical molecules like hydrogen peroxide are generated during normal metabolic processes into the cells and oxidative stress is acquired due to either excess production of FRs or shortage in antioxidant enzymes such as SOD, CAT, and GSH-Px [12-14]. Free radicals devastate polyunsaturated fatty acids of cell membrane lipids and causes lipid peroxidation which is used as determinant of oxidative stress and cellular injury indicator [15,16]. Malondialdehyde (MDA), one of the lipid peroxidation by-products, is considered the most abundant and reliable biomarker either in assessment of lipid peroxidation or indirect detection of FRs levels [17-19]. MDA is merged with cell membrane and leads to intracellular damage, cross-linking with phospholipid and enzymes [20,21]. Consequently, overwhelming of antioxidant defence is believed to be the trigger of broad difference processes on various organs which are conducive to diseases and tissue damage [22,23]. Determination of antioxidant enzymes in fish diseases has been performed in various studies [23-25] and catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px have been determined in most fish species [23,26]. Recently, one of the other antioxidant enzymes which has been paid more attention, is PON. It synthesized in liver and liberated into blood and belongs to esteric hydrolases that hydrolysis xenobiotics such as organophosphates. Furthermore, PON alleviate oxidative stress in tissues and cells and its activity has been reported in fish [27,28].

To our knowledge, this is first study to evaluate alterations of some blood parameters such as sialic acid (as inflammatory marker), oxidative stress indices and other parameters along with histopathology in *Oncorhynchus mykiss* saprolegniasis and likely some of them be effective in the management of saprolegniasis in Rainbow trout.

MATERIAL and METHODS

Examination of Saprolegniasis

This study was conducted in Urmia city during 2014-2015, Iran. Fish with lack of any internal and external parasites along with lack of bacterial infection which examined with clinical signs were selected for the study. Water quality consists of normal conditions such as water temperature 11°C heat low C, pH 7.2, oxygen concentration 6.8 mg/L. Three hundred and fifty-eight rainbow trout were assessed from eight aquaculture farms of Urmia and among them, one hundred and seven saprolegniasis rainbow trout without UDN (ulcerative dermal necrosis) disease symptoms were detected by white or gray patches on the head and fin with cotton-like appearance and were diagnosed by GYPS agar plates (Glucose-Yeast-Peptone agar) which is specific for *Saprolegnia* culture [29]. Also, the same number fish that were examined for lack of any saprolegniasis and other diseases (parasitic, viral and bacterial) signs were selected as the healthy group. This study was confirmed with the ethical approval (No: 0552762948126) in the Ethics Committee of Urmia Islamic Azad University.

Preparation of Blood Samples

Anesthesia was not performed on the fish because it can induce adverse effects on blood parameters [30]. Blood samples were collected from all fish via caudal vein into EDTA-contained tubes for hematologic tests and plasma preparation. Thereafter, all tubes were centrifuged with 4000 RPM for 10 min at room temperature.

Hematology and Plasma Chemistry

Standard micro-hematocrit method was carried out for packed cell volume (PCV) determination. Hemoglobin (Hb) measurement was determined according to cyano-methemoglobin method with spectrophotometer at 540 nm absorbance. White blood cells (WBC) and red blood cells (RBC) count were carried out with Dacie`s solution as a diluting fluid [31]. Determination of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) as the erythrocyte indexes were detected by Haney et al.[32] method. Moreover, blood smears were stained with Giemsa solution 5% for differential count of leukocyte and blood parasites examination and specification of each cell percentage were counted in one hundred cells. TSA was determined by Sydow [33] method (spectrophotometric, Spekol-1500). SOD, CAT, GPx activities were assessed in erythrocyte hemolysis on the basis of manual methods, Abei [34] for CAT and commercial kits (Randox laboratories Ltd. G.B) for SOD, GSH-Px and total antioxidant capacity (TAC), (Auto analyzer, Alcyon-300, USA) as well as, GL, Urea, CREA, TPP, AST, ALT detected by commercial kits (Pars azmoon., Chemical co., Tehran, Iran) with Auto analyzer, Hitachi- 917, Japan. Finally, MDA levels and PON activity was detected by Satoh [35] and Furlong [36] method respectively (spectrophotometer, model Cecil, Italy).

Histopathological Examination

Tissue specimens of healthy and infected fish from liver, spleen and kidney were taken and rapidly fixed in neutral buffered formalin 10%. Thereafter, conventional paraffin wax embedding technique was performed in

fixed specimens. Then, the sections were cut 5 microns in thickness and were stained by Hematoxyline and Eosin (H&E) and Periodic Acid Schiff (PAS) staining methods.

Statistical Analysis

Statistical analysis was accomplished in all analyses. Mean ± SD and determination of variation between the data results were carried out with Student's *t*-test with SAS version 9.1 and significance level was specified at (P<0.01).

RESULTS

Saprolegniasis causes significant alterations in the majority of plasma biochemical profiles of infected fish compared to healthy ones (P≤0.01), which was documented in *Table 1*. Moreover, hematological values assessment was revealed in *Table 2*. According to the hematological results, HCT, Hb amount and RBC count decreased (P≤0.01). Neutrophilia, lymphopenia, monocytosis and eosinophilia occurred in saprolegniasis group than healthy ones. In respect of nitrogen metabolism, an increase in Urea and CREA and TPP was observed. In comparison with healthy ones, marked decrease of GL was exhibited in diseased fish. ALT and AST concentration as the liver function indices were different, there was significant elevation in AST with no remarkable alterations in ALT. High levels of TSA and MDA were determined in infected ones than healthy group and concentration of antioxidant enzymes considerably reduced. In the case of SOD, there are no significant alterations were detected.

Histopathologic results of this present study show that cutaneous saprolegniasis in Rainbow trout causes three lesions in different tissues. These are as follows: Liver: focal necrosis, edema, the increase of melanomacrophage centers, fatty change and glycogen storage state *(Fig. 1, Fig. 2)*. Spleen: the increase of melanomacrophage centers *(Fig. 2)*. Kidney: Tubular vacuolar degeneration, glomerular atrophy and partly increase of melanomacrophage centers *(Fig. 3)*.

Table 1. *The following table reveals all parameter alterations in patient compared with control group*

Tablo 1. *Aşağıdaki tablo, tüm parametre değişikliklerini hasta grubun kontrol grubu ile karşılaştırıldığında ortaya çıkarır*

Parameters	Control Group	Patient Group
TSA (mg/dl)	39.26±4.03	57.63±3.21†
MDA (nmol/ml)	6.49±1.417	8.84±0.922†
PON (U/L)	79.72±22.585	42.64±10.164†
SOD (U/gHb)	1569.66±214.719	1789.31±769.025
CAT (k/gHb)	102.70±13.547	61.85±8.102†
GSH-Px (U/mgHb)	98.35±11.086	53.48±9.294†
TAC (mmol/L)	0.86±0.7	0.47±0.11†
GL (mg/dl)	78.24±5.861	56.92±5.943†
CREA (mg/dl)	1.172±0.328	3.01±0.296†
UREA (mg/dl)	6.32±1.573	12.6±2.236†
TPP (g/dl)	5.03±0.367	5.85±0.506†
AST (U/l)	179.64±55.3164	247.32±21.7595†
ALT (U/l)	56.92±19.7081	68.56±18.6303

Data are expressed as mean ± standard deviation; † Significantly different from the control group (P<0.01)

Table 2. *The following table denotes hematological alterations in saprolegniasis group compared with healthy ones*

Tablo 2. *Aşağıdaki tablo, hematolojik değişiklikleri Saprolegniasis grubunda sağlıklı olanlarla karşılaştırıldığında gösterir*

Parameters	Control Group	Patient Group
PCV	42.68±3.14	30.8±2.34†
Hb	9.24±0.73	6.25±0.84†
RBC	3.09±0.46	1.26±0.15†
MCV	289.99±41.15	277.04±17.79
MCH	66.76±7.65	67.12±3.86
NEU	18.48±2.16	30.80±2.04†
LYM	69.68±1.84	55.44±5.23†
MONO	2.60±1.41	6.36±1.91†
EOS	5.48±1.29	10.92± 4.15†

Data are expressed as mean ± standard deviation; † Significantly different from the control group (P<0.01)

DISCUSSION

Sialic acid (SA) is known as a valuable marker for diagnosis and prognosis of inflammatory diseases. Motoi et al.[37]. In this study, SA levels increased in infected group than non-infected ones. SA concentrations have been specified high during the course of many diseases. Citil et al.[11]. We could not find any published studies about SA levels in rainbow trout saprolegniasis. In these circumstances, rise in TSA may be attributed to acute phase proteins elevation, because acute phase proteins, such as α1-acid glycoprotein, are sialated glycoproteins [37]. The another probable reason can be ascribed to inter-relationship between SA and innate immune system of skin mucous. Fish skin mucous is involved as first line of defence against pathogens [38]. The multiple components of innate immune system are aggregated in fish skin mucous, such as glycoproteins, lysozyme, immunoglobulin, anti-microbial peptides, lectins, C-reactive proteins [39]. Besides, skin mucous secretions contain sialic acid in various fish species and sialated glycoproteins constitute in skin mucous ingredients [40]. They involve in restrain and protection against bacterial break down and viral invasion [41]. Since SA is firmly bounded to bacterial macromolecules and bacteria, thereby impede the adherence of pathogen agents to epithelial cells [42]. It is likely that sialic acid of skin mucous interacts with *saprolegnia spp* for hindering its extension. Therefore, SA increases in blood for compensation of SA consumption in skin mucous.

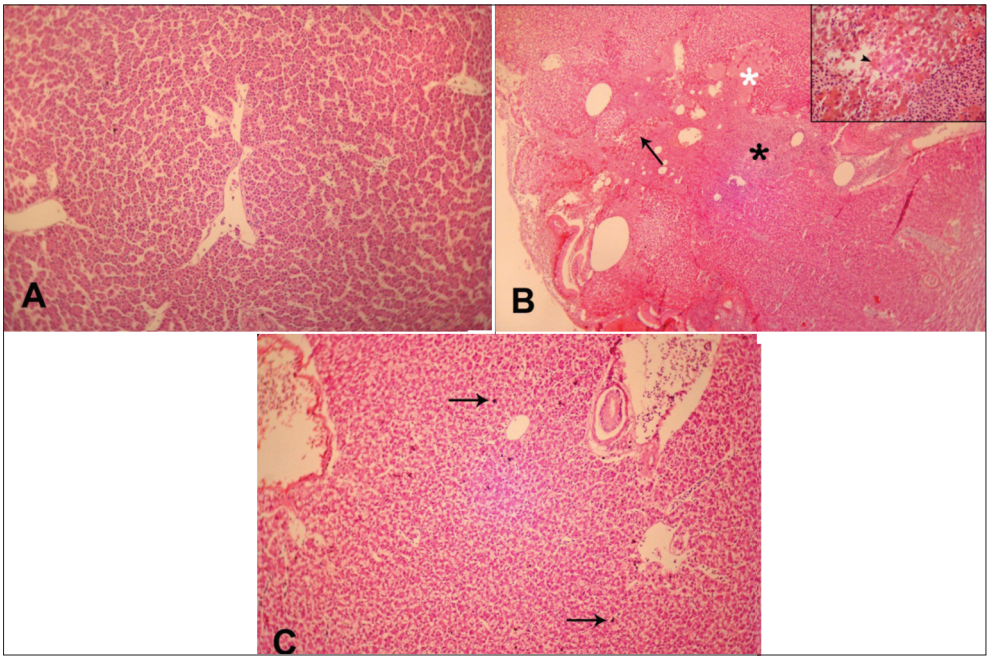

Fig 1. A. Control group: Hepatic tissue is normal and clear vacuoles in hepatocytes are very little, (H&E), X200; **B.** Cutaneous saprolegniasis group: (Main figure): Focal edema with serosal fluids *(white asterisk)* in liver, hemorrhage and focal infiltration of lymphocytic cells *(black asterisk)*, local hepatic necrosis *(arrow)*, (H&E), X80. (Inner figure): Necrotic hepatocytes containing eosinophilic cytoplasm and destructed nuclei *(arrowhead)*, (H&E), X800; **C.** Cutaneous saprolegniasis group: Increase of clear vacuoles in most of hepatic cells is seen. Dispersion of Melanomacrophage centers has increased in hepatic tissue *(arrow)*, (H&E), X200

Şekil 1. A. Kontrol grup: Karaciğer dokusu normaldir ve hepatositlerde net vakuoller çok az vardır, (H&E), X200; **B.** Deri Saprolegniasis grubu: (Ana Şekil): Karaciğerde serozal sıvıları ile fokal edema *(beyaz yıldız)*, kanama ve lenfositik hücre odak infiltrasyonu *(siyah yıldız)*, lokal hepatik nekroz *(ok)*, (H&E), X80. (iç şekil): Nekrotik hepatositler ki içinde eozinofilik sitoplazma ve tahrip olmuş çekirdek var *(okbaşı)*, (H&E), X800; **C.** Deri Saprolegniasis grubu: Karaciğer hücrelerinin çoğunda açık vakuoller artışı görünür. Melanomacrophage merkezlerinin dispersiyonu karaciğer dokusunda artmıştır *(ok)*, (H&E), X200

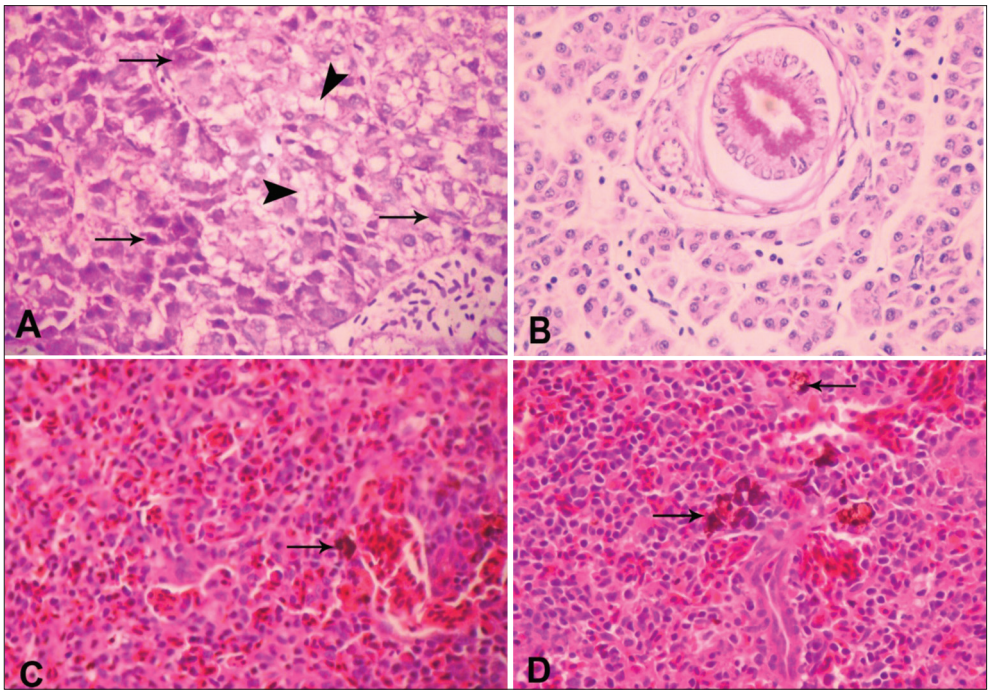

Fig 2. A. Cutaneous saprolegniasis group: Focal fatty change *(arrowhead)* and cytoplasmic aggregations of glycogen in hepatocytes *(arrow)*, (PAS), X960; **B.** Control group: Normal appearance of hepatic tissue, (PAS), X960; **C.** Control group: Normal splenic tissue containing few melanomacrophege centers *(arrow)*, (H&E), X960; **D.** Cutaneous saprolegniasis group: Hyper activation and increase of melanomacrophage centers *(arrow)* in spleen, (H&E), X960

Şekil 2. A. Deri Saprolegniasis grubu: Fokal yağlı değişim ve hepatositlerin sitoplazmasında glikojen toplamaları *(ok)*, (PAS), X960; **B.** Kontrol grubu: Karaciğer dokusunun normal görünümü, (PAS), X960; **C.** Kontrol grubu: Normal dalak dokusu içeren birkaç melanomakrofaj merkezleri *(ok)*, (H&E), X960; **D.** Deri Saprolegniasis grubu: Hiperaktivasyon ve dalakta melanomacrophage merkezlerinin artışı *(ok)*, (H&E), X960

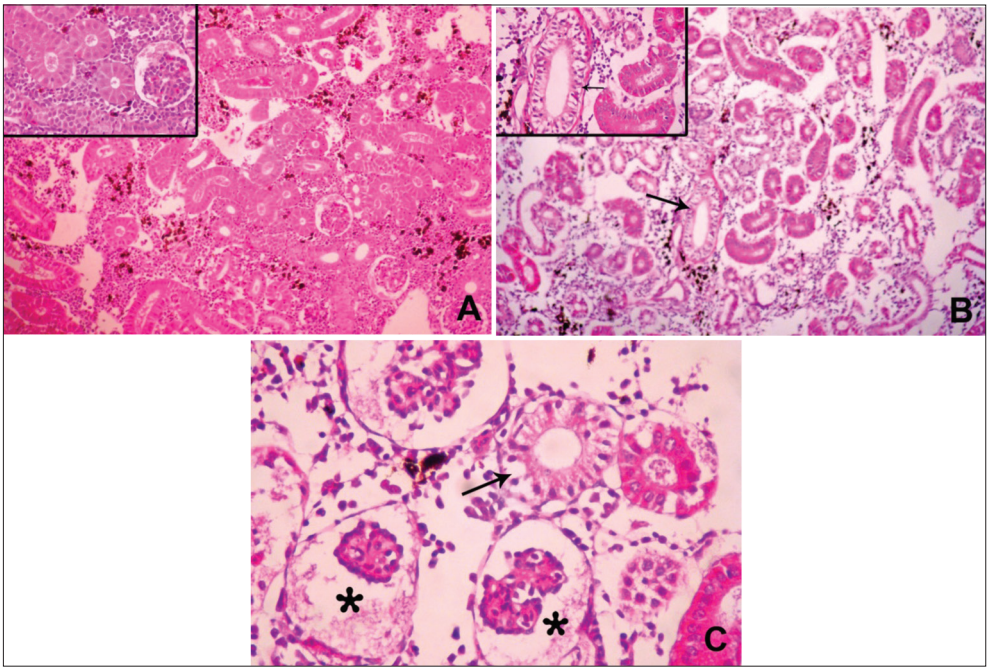

Fig 3. A. control group: (Main figure): Normal appearance of renal glomeruli and tubules, (H&E), X240. (Inner figure): Higher magnification of normal kidney, (H&E), X960; **B.** Cutaneous saprolegniasis group: Vacuolar degeneration in some of renal tubules *(arrow),* (Main figure): (H&E), X240. (Inner figure): (H&E), X960. **C.** Cutaneous saprolegniasis group: Glomerular atrophy and cystic formation *(asterisk)* and tubular vacuolar degeneration *(arrow).* (H&E), X960

Şekil 3. A. Kontrol grubu: (Ana şekil): Böbrek glomerül ve tübülün normal görünümü, (H&E), X240. (iç şekil): Normal böbrek üstü büyütme. (H&E), X960, **B.** Deri Saprolegniasis grubu: Vakuolar dejenerasyon bazı böbrek tübüllerinde *(ok),* (Ana şekil): (H&E), X240. (iç şekil): (H&E), X960; **C.** Deri Saprolegniasis grubu: Glomerüler atrofi ve kistik oluşumu *(yıldız)* ve tübülar vakuolar dejenerasyonu *(ok),* (H&E), X960

Enhancement of Urea and Crea in *oncorhynchus mykiss* have been revealed in viral haemorrhagic septicemia (VHS) [43] and after seven-day post-infection of *Tilapia nilotica* with *saprolegnia parasitica* [4] which may be attributed to kidney insufficiency due to the infestation of *Saprolegnia* hyphae. Versus, Rehulka [43] reported Crea decrease in Atlantic salmon vibriosis. In the present study, high nitrogen metabolism values (Urea and Crea) in infected ones can be attributed to saprolegniasis induced-kidney insufficiency. Infected fish exhibited an increase in TPP concentration compared to healthy ones. TPP is considered as health index in fish medicine and some factors induce hyperproteinemia, such as metabolism severity, nutrition, health status and high stocking density [44,45]. This might have been the case in our study as high TPP is attributed to fluid volume disorders, overcrowded stockings and/or elevation of acute phase proteins due to saprolegniasis. In the present study, AST increase was accompanied by no significant alterations of ALT, which may suggest effects of fungus toxins or presence of *Saprolegnia spp* hyphae in liver. Also, histopathological results confirm liver damage. High activity of ALT was exhibited in Atlantic Salmon Vibriosis, Rehulka [43] but Zaki et al.[4] reported AST increase in *Tilapia* nilotica affected *Saprolegnia parasitica*. Moreover, bacterial infection of Rainbow trout such as *Aeromonas salmonocida* contributes in significant elevation of transaminases [43]. Plasma MDA as an indicator of lipid peroxidation and oxidative stress index, Elia et al.[24] also significantly increased in present study. We did not find any study about plasma MDA changes in fish fungal diseases. It suggests the production of free radicals and lipid peroxidation. Arabi and Alaeddini [26] and Vutukuru et al.[20] revealed MDA increase in *Oncorhynchus mykiss* gill hemogenate and visceral tissue of *Esumus danricus*, respectively. However, some studies have reported low levels of MDA in rainbow trout and Teleost [25,46] which is not in accordance with our study. In recent study, antioxidant enzymes (CAT, GSH-Px and TAC) were determined to be low. It postulated that their impression on oxidative stress lead to alleviation and/or is ascribed to low nutrition conditions. Mathew et al.[47] indicated significant reduction of CAT, GSH-Px and TAC in infected *Penaeus monodon* with white spot syndrome virus (WSSV) and Castex et al.[18] reported decrease of CAT and GSH-Px activities in affected *Litopenaeus stylirostris* with *Vibrio nigripulchritudo* infection. There was no significant change in SOD activity which agrees with unchanged liver SOD activity in affected rainbow trout with copper rich foods. Knox et al.[48]. However, SOD alterations have been investigated in some studies [25,49]. The GL concentration as the main analyte of carbohydrate metabolism accompanied with significant decrease. This may have been the case in our study as saprolegniasis causes anorexia and/or induction of glycogenesis (glycogen accumulation) in liver, which is confirmed with histopathological findings (PAS staining). We could not find any information regarding possible link

between plasma GL with glycogen storage in rainbow trout saprolegniasis. Pescador et al.[50] revealed relevance of hypoxia with glycogen accumulation and indicated that hypoxia-inducible factor causes glycogen synthase promotion in human muscle as well as hypoxia induce steatohepatitis in mice [51]. Since, based on Brauner and Wang study [52], hypoxia arises during anaemia. Therefore, it is possible that anaemia-induced hypoxia leads to glycogen accumulation along with fatty changes (steatohepatitis) in liver of saprolegniasis group. Furthermore, bacterial infections and VHS diminish GL concentration in rainbow trout while some of diseases such as overcrowding stress, heavy metals, herbicides and pesticides cause GL enhancement [43,45,53,54]. PON plays a vital role in Xenobiotic biotransformation and protects against lipid peroxidation [55,56]. Many studies reported PON activity in human [57], but, no comprehensive studies have been done regarding PON in veterinary medicine. In this study, PON concentration was found to be low. Most probably PON involves as inhibitory role on lipid peroxidation reaction. In this study, saprolegniasis caused marked alterations in hematology parameters than healthy ones including low values of PCV, RBCs count and Hb without significant changes in MCV, MCH, which indicated anaemia. Low count of peripheral RBC may associate with lose of hemapoietic tissue. Erslev [58] reported concurrent incidence of chronic renal failure along with uremia which is regularly accompanied by anaemia and its intensity is correlated with uremia severity. It is worth noting that many published studies have reported induction of anaemia in salmonids with bacterial and fungal infection [43,59]. It is postulated that kidney insufficiency-mediated uremia is caused anaemia in saprolegniasis group. Zaki [4] also pointed out correlation between plasma cortisol level with PCV, Hb and RBC reduction in *Tilapia nilotica*. It is more likely that occurrence of high cortisol level through disease induced- stress plays another cause in decrease of PCV, Hb and RBC with above mentioned mechanism in saprolegniasis. The significant increase of WBCs such as neutrophil, eosinophil and monocyte with lymphocyte decrease were noticed in the present study, which denotes cellular-immunity system interaction with fungal infection, Jamalzadeh [59]. Also, lymphopenia is attributed to three different mechanisms. Firstly, stress induced lymphopenia with following mechanisms, lymphocyte re-dispensation to lymphoid organs, cell destruction or decrease in blood circulation due to high levels of cortisol. Secondly, hypoxia-induced lymphopenia and thirdly, lymphocyte infiltration to tissues which it has been documented in *Fig. 1-B* [60-62].

Histopathological process carried out in liver, kidney and spleen. The results suggest that saprolegniasis-induced hypoxia may involve in glycogen accumulation and fatty changes into hepatocyts, second, the observed liver and renal damage could attribute to invasion of *saprolegnia* spp hyphae. In spleen, the increase of melanomacrophage centers usually occurs through antigens confrontation. According to the comparison of histopathologic results of control group with saprolgniasis ones, it indicated that the increase of melanomacrophage centers in spleen may ascribe to contamination by *Saprolegnia* spp.

In conclusion, these results showed the saprolegniasis impresses on blood parameters of rainbow trout with histopathological changes in different organs. These findings may persuade attempts to expand the importance of biochemistry and clinical hematology in the health screening programs of the rainbow trout in intensive aquaculture.

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