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EFFECT OF OZONATED WATER TREATMENT ON CLINICAL SIGNS, SURVIVAL RATE AND HISTOPATHOLOGICAL ALTERATIONS IN COMMON CARP, *CYPRINUS CARPIO* L. INFECTED WITH *SAPROLEGNIA SPP.*

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ABSTRACT

Present study was undertaken to assess the efficacy of Ozone and formaldehyde (as a reference treatment) on controlling Saprolegniasis in common carp, *Cyprinus carpio* L. *Saprolegnia spp*. were isolated on special culture media for fungi from 50 infected specimens of fishes were identified as *Saprolegnia spp*. Viable fungal suspension of Saprolegnia was determined and adjusted at a concentration of 2×10^4 zoospores l^{-1} . To control this fungus, a total of 120 common carp weighing 80±10g were randomly distributed into six replicated groups (10 fish/replicate) and were treated as follows; C-: control healthy without treatment; C+: control infected with *Saprolegnia spp.* without treatment; T1, T2 and T3: fish were infected with *Saprolegnia spp*. and treated with Ozone 0.25, 0.50 and 0.75 mg/l per hour respectively; T4: fish were infected with *Saprolegnia spp.* and treated with formalin 0.15 ml/ l for 30 min for 3 successive days. Clinical signs and survival rate were studied. After 14 days of treatment with Ozone, samples were collected from fish for histopathological studies. Among the Ozone treatment 0.50 mg/l showed highest survival rate (90%), survival rate of the control group (without disinfectant) was 20%. Histopathological studies revealed significantly increased ($p<0.05$) percentage of gill epithelial proliferation and epithelial lifting, also fusion of the secondary lamellae, in fish from ozonated groups relative to C+ and C- groups. However, there were no significant differences in histopathology frequency/severity among the ozonated groups (T1, T2, T3 and T4). Skin of C+ group exhibited severe histopathological alterations including sloughing, erosion and ulcerative of epidermis penetrating up to dermal tissue. While Ozone treatment groups showed increase number of mucous cells and MNCs infiltration. Ozone appears to be a valuable disinfectant against *Saprolegnia* infection; at the dose of 0.50 mg/l. In conclusion, the results indicated the efficacy of Ozone as antifungal in controlling Saprolegnia infection. Thus, Ozone could be used as potential and promising alternatives to chemotherapeutics compounds in aquaculture, to achieve sustainable, economic, and safer fish production.

Keywords: *Cyprinus carpio, Ozone, Saprolegnia, Saprolegniasis.*

INTRODUCTION

Saprolegniasis is one of the most problematic mycological diseases in fresh water fish This disease caused by species in the genus *Saprolegnia***.** It causes significant economic problems in the fish culture system and can be also extensively destroy fish eggs in hatcheries (Bly *et al.*, 1992; Pottinger and Day 1999; Hussein *et al.*, 2001). Fungal infection of fish by oomycetes commonly known as water molds (Davis, 1953; Duijn, 1973). This fungus produces spores and these spores which readily spread disease (Hussein *et al.*, 2001). Saprolegniosis is recognized by a relatively superficial, cottony/woolly, white to brownish fungal growth over the body surface, fins, head region or in gills, or on fish eggs when in water. Fungal growth could pentrating up to dermis layer and to the musculature layer with time with sloughing and desqumation of the epidermis (Van West 2006).

Despite synthetic antifungal are available and are applied to control the disease, their unsystematic use of these agents causes environmental threat. Although, the use of these chemicals are effective in controlling fungal infection, but are no longer recommended which tend to limit the usage. Higher dosage or the development of new chemicals to replace those to which fungi are resistant besides the negative impact on the immune system and accumulate in the tissue residues (Van West, 2006). Secondly, some fungicides are not easily biodegradable and tend to persist in the environment. Hence, it was necessary to get alternative approaches to address the problems related illnesses farms and move away from the use of chemicals that may cause environmental problemst (Khoo, 2000). Among those alternative and modern methods of technology ozone therapy for the control of diseases and reduce damage and scaled the heavy losses in fish farms (Bullock *et al.*, 1997).

It has shown the positive effect of ozone treatment against infection in abalone (Dixon *et al.*,1991) and against viral pathologies of the pancreas in Atlantic salmon (McLoughlin *et al.*, 1996) as well as in crustaceans, against viral infection (Chang *et al.*, 1998). Tipping, (1988) also showed the bene-

ficial effect of in the treatment of ceratomyxosis in rainbow trout. In the back drop of above information, there is a need for further research to identify and control the *Saprolegnia spp.* using modern strategy such as Ozone. Hence, the aim of this work is to study the efficacy of ozone treatment on clinical signs, survival rate and histopathological alterations in common carp infected with *Saprolegnia spp*.

MATERIALS AND METHODS

Isolation and Identification of *Saprolegnia spp.*: A total of 50 fish with an average weight 150-250 g were collected from local cages (dimensions of the cage 3×4m with the depth of 2m) from aquaculture in Diyala province/Iraq (Fish were showed skin lesions (cottony/woolly, white growth) like fungoid lesion and ulcerations on body and were transferred to the laboratory for fungal isolation. Cultures were prepared on Sabouraud Dextrose Agar (SDA). Growth was observed by incubating them for 3-5 days at 20˚C. After the incubation period, all pure colonies were inspected for morphological characteristics and microscopical features. For identification, slides were prepared from each colony by picking up small tuft of mycelium and were stained with Lacto phenol cotton blue and examined under high magnification of microscope. *Saprolegnia* was shown under microscope filamentous mycelium, hyphae were hyaline and coenocytic. Identification of *Saprolegnia* was carried out according to Cayla (2014). Viable fungal suspension of *Saprolegnia* was determined and adjusted at a concentration of 2×10^4 zoospores l^{-1} using haemocytometer (Horwitz *et al.,* 1975). Then, the fungi isolates were introduced to the fish tanks (2×10^4 zoospores per l) and left in the fish tanks for one week. When signs of Saprolegnia *Saprolegnia* growth were evident on the fish (cottony/wooly like appearance), different concentrations of the ozone (0.25, 0.50 and 0.75 mg/l) were introduced into aquarium tanks.

Preparation of dissolved Ozone: The ozone was generated in the water using electrical corona discharge method three devices were used to generate ozone with different concentrations (0.25 and 0.50 and 0.75 mg /l). Seat devices near the glass basins and reached the main aperture of each device a flexible tube of synthetic rubber to the bottom of treatment basins, all of a rubber tube ends with diffuse stone. The concentrations of dissolved ozone count and periods of device drivers, linked devices, electric control system included three- regulation for the timing of flash timers, programmed intervals devices to run on demand to be (0.5, 1, 1.5) minutes per hour concentrations (0.25 and 0.50 and 0.75 mg/l), respectively.

Experimental Design: About 120 healthy fish of *C. carpio* of body of mean weight 80±10 g was brought from a commercial fish farm from Hilla province or Babylon city province, Iraq. Fish were transported in plastic tanks aerated with air pumps. After that, fish were acclimatized for two weeks prior in laboratory conditions. Fish were randomly distributed into six replicated group (10 fish/replicate), ten fish in each glass aquaria of (measuring 40 x 50 x 70 cm) dimensions. Fish were kept in chlorine free tap water supplied, fishes were fed with commercial feed pellets at 2% body mass twice daily. The fish were maintained at a natural photoperiod 12 h light /12 h dark. The chemo-physical parameters of the water were measured during the experimental period as follows: Temperature 22 ± 1 ºC, Dissolved O_2 6.10± 0.5 mg l^{-1}, pH 7.10 ± 0.05. Next, Fish were treated as follows: C-: control healthy without treatment; C+: control infected with *Saprolegnia spp.* Without treatment; T1, T2 and T3: fish were infected with *Saprolegnia spp*. and treated with Ozone 0.25, 0.50 and 0.75 mg/l per h respectively; T4: fish were infected with *Saprolegnia spp.* and treated with formalin 0.15 ml/ l for 30 min for 3 successive days.

Clinical Examination and Survival Rate: About 120 living/dead fish were monitored for abnormal behaviors and external lesion over the body surface, gills and eye according to the method described by Amlacker (1970). Percentage survival was calculated using the following equation:

Survival rate (%) =final number of fish survivor/initial number of fish stocked×100

Histopathological Study: Histological study were carried out as described by Myers *et al.,* (1998). Selected tissues (skin with muscles and gills), were immediately fixed in 10% formaldehyde solution for 48 -72 h. The tissues were then processed routinely and prepared into paraffin blocks. The blocks of the tissues were cut (5-7 µm thickness) and stained with Haematoxylin and Eosin (H&E) and skin sections were stained with Periodic Acid Schiff (PAS) to show the fungal hyphae. Slides were examined using light microscopy and photographed using Optika Vision Microscopy Digital UBS camera. Detailed descriptions of pathology were done for the experiments according to Bernet *et al.,* (1999). For the gill sections, histological features were determined, measured when appropriate and scored relative to the lamellae number. The secondary lamellae that

were complete from tip to base were involved for quantitative analysis according to Mustafa (2012).

Statistical analysis: Statistical analysis was achieved using Sigma Plot v11.0 software. A quantitative assessment histopathological investigation was done through One-way analysis of variance (ANOVA) to determine the significant differences between variables. A probability level equal or less than 5% ($P < 0.05$) were considered significantly different.

RESULTS

Isolation and Identification of *Saprolegnia spp.*

Macroscopically and Microscopically: Initially, lesions were appeared as small, rounded, depigmented regions, sometimes with hemorrhagic borders. In advanced stages lesions developed to ulcerative area, penetrating via the skin and into the musculature tissue, and almost the fish almost were entirely covered with abundant fungal growth. The morphological criteria of the growth of fungal colonies on SDA are appeared after 24-72 h from incubation at 20ºC as circular mass of filaments, whitish in color and brownish in the center and characterized by an extensive and dense mycelium (Figure 1).

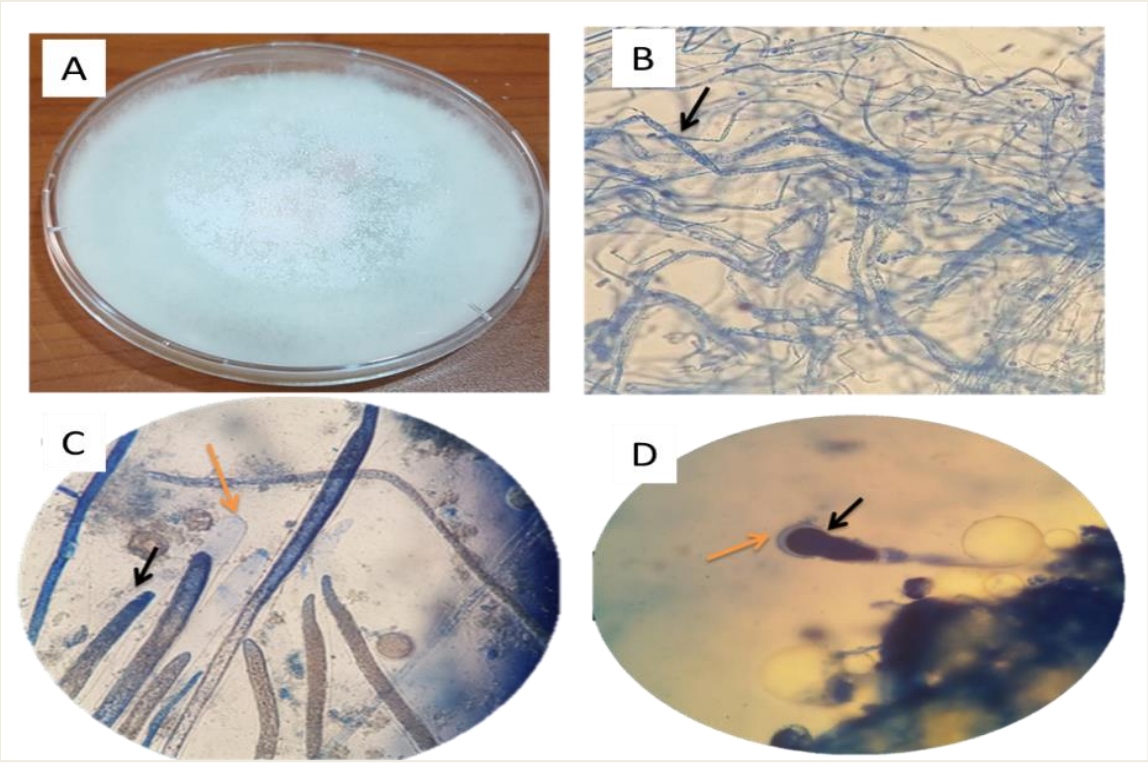

Figure 1: A- *Saprolegnia spp.* cultures on SDA at 20ºC for 3-4 days started as long hairs with whitish cottony color. **B-** The wet smear of skin showing masses of mature and immature sporangia filled with huge number of sporangiospores. **C&D** The hyphae looked profusely, separated and were non-septated, these morphological features were representative of the *Saprolegnia spp.*, stained with Lacto-phenol cotton blue. 400 X.

Clinical Signs and Survival Rate: The results of clinical sings and survival rate for all treatment groups are elucidated in Table1. The main clinical sings on C+ group after 3-5 days from infection with *Saprolegnia spp.* were appearance of filamentous strands called hyphae, it starts off on the back of the fish as circular patches which get bigger and spread all over the body of fish approximately 40% of the body surface were covered. After 4-6 days from infection some cases became ulcerative, the hyphae were penetrating through the skin into the muscular tissue, and the fishes are completely covered with thick fungal growth. Mortality rate reached up to 80% at the end of experimental period (i.e., after 14 days). Ozone treatment showed a good result for disappearance of fungal growth and clinical signs on infected fish in aquaria especially at a dose of 0.50 mg/ l (T2) and at 0.75 mg/l (T3) for 7 successive days and with survival rate reached up to 90% and 60% respectively. While, in T1 the survival rate was 40%, the growth of hyphae was disappeared after 4 days from starting the treatment with ozone, the whitish patching was disappeared after 7 days from experimental period, the color of the skin is returned to normal after 10 days from the experimental period. Whereas, the growth of hyphae in T2 were disappeared after 2 days from starting the treatment, the whitish patching was disappeared after 4 days from experimental, the color of skin

was returned to normal after 7 from experimental period. The survival rate was 90%. T3 the growth of hyphae was disappeared after 2 days from starting the treatment, the whitish patching was disappeared after 4 days, the color of skin was returned to normal after 7 days from experimental period. The survival rate was 60%. The growth of hyphae was disappeared after 4 days from starting the treatment in T4, the whitish patching was disappeared after 9 days from experimental period, the color of skin returns to normal after 11 days from experimental period and the survival rate reached up to 80%.

Table 1: Clinical sings and survival rate for all treatment groups of *C. carpio* infected with *Saprolegnia spp.* and treated with different concentrations of Ozone.

Groups	No of fish	Follow up through 14 days		Fungal growth and clinical signs	Survival rate (%)
		Dead	Survive		
C-	20	0	20	- - -	100
C+	20	16	4	+++	20
T1	20	14	6	+ + / -	40
T2	20	2	18	- - -	90
T3	20	6	14	- - -	60
T4	20	4	16	- - -	80

+: signs still occur - : signs disappear

Histopathological study

Gills section: The gill tissues from control group exhibited normal organization pattern of primary and secondary lamellae. The lamellae are covered by epithelial cells (Figure 2 A). The main histopathological changes noted after 14 days from infection with *Saprolegnia sp*. in gills sections were in C+ group, including: epithelial lifting, hyperplasia of epithelial cells, edema in the filament, dilation of the central venous with blood congestion and necrosis (Figure 2 B-D). These changes were lesser in extent in ozone treatment groups (T1, T2, T3 and T4) (Figure 2 E&F). For the majority of these detected lesion types, there w no significant difference in histopathology severity/frequency among the ozonated groups; however, fish from ozonated water and treated with formalin (T1, T2, T3 and T4) had statistically higher ($p<0.05$) levels of gill epithelial proliferation and epithelial lifting, also fusion of secondary and primary lamellae relative to C+ and C- groups (data not shown; ANOVA $p<0.05$).

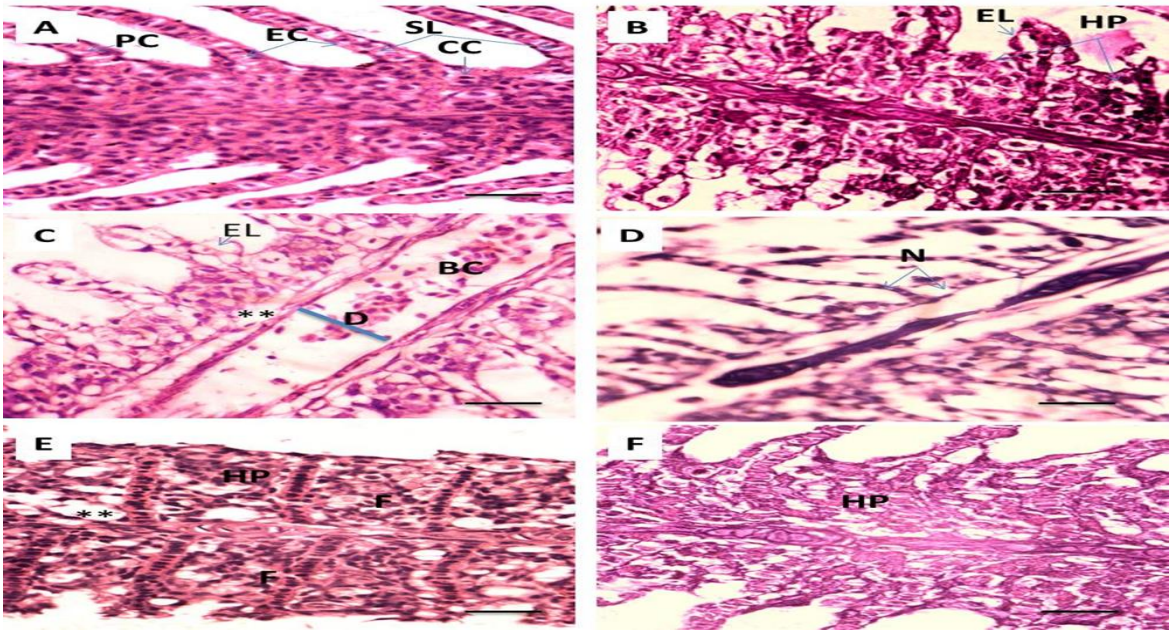

Figure 2: Histopathological changes in gills sections from control and ozone treated groups of *C. carpio.* (A): Control gill, showing normal arrangement pattern of the secondary lamellae (SL), epithelial cell (EC), pillar cell (PC) chloride cell (CC). (B-D): Positive control infected with *Saprolegnia* showing epithelial lifting (EL), hyperplasia of the epithelium (HP), edema (**) in the filament, dilation of the central venous (D) with blood congestion (BC) and necrosis. (E&F): gills infected with Saprolegnia and treated with ozone and formalin showing epithelial lifting (EL), hyperplasia of the epithelium (HP), interstitial edema (**) in the filament. H&E stain; Thickness 5-8µm. Scale bars 50µm.

Skin Section: Control group (uninfected fish) showed normal histological structure of skin layers (i.e. epidermis, dermis, basal layer, stratum compactum and muscular layer) (Figure 3 A). While, positive control exhibited several histopathological alterations including: complete erosion and ulcerative of epidermis penetrating up to dermal tissue associated with mononuclear cells (MNCs) (monocytes/macrophages and lymphocytes) infiltration, increase number of alarm cells, deposition of sub epidermal thick fibrous material (Figure 3B&C). In addition, some sections showed sloughing and destruction of epidermal tissue and in some there are complete loss of epidermal layer with cellular debris and fungal hyphae, severe vacuolation filled with fungal material which could be hyphae of the Saprolegnia (Figure 3 D&E). Whereas, the skin of all ozonated groups (T1, T2, T3) showed increase number of mucous secreting cells with MNCs infiltration (Figure 4 A&B). However, T4 revealed severe dermal necrosis with intramuscular edema, increase number of melanophores and MNCs infiltration (Figure 4 C & D).

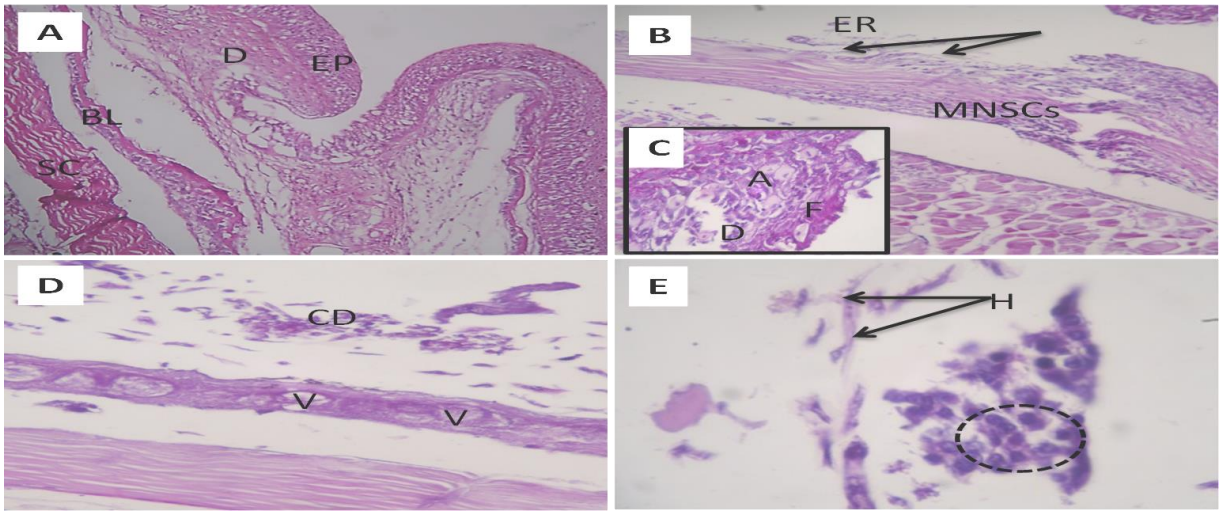

Figure 3: Photomicrograph sections showing histological structures through skin of *C. carpio* infected with *Saprolegnia spp.* and treated with Ozone **(A):** control skin showing epidermal layer (EP), basal layer (BL) and stratum compactum (SC) 10x; **(B-E)** positive control exhibiting complete erosion and ulcerative of epidermis (ER), associated mononuclear cells infiltration (MNCs); (**C**) increase number of alarm cells (A), deposition of fibrous material (F) with sloughing and destruction of epidermal tissue; (**D**) showing complete loss of epidermal layer with cellular debris (CD) mixed with fungal hyphae with severe vacuolation filled with fungal material (V); **(E)** showing fungal hyphae (H) with cellular infiltration (black circle). PAS stain; Thickness 5-7µm. 400x.

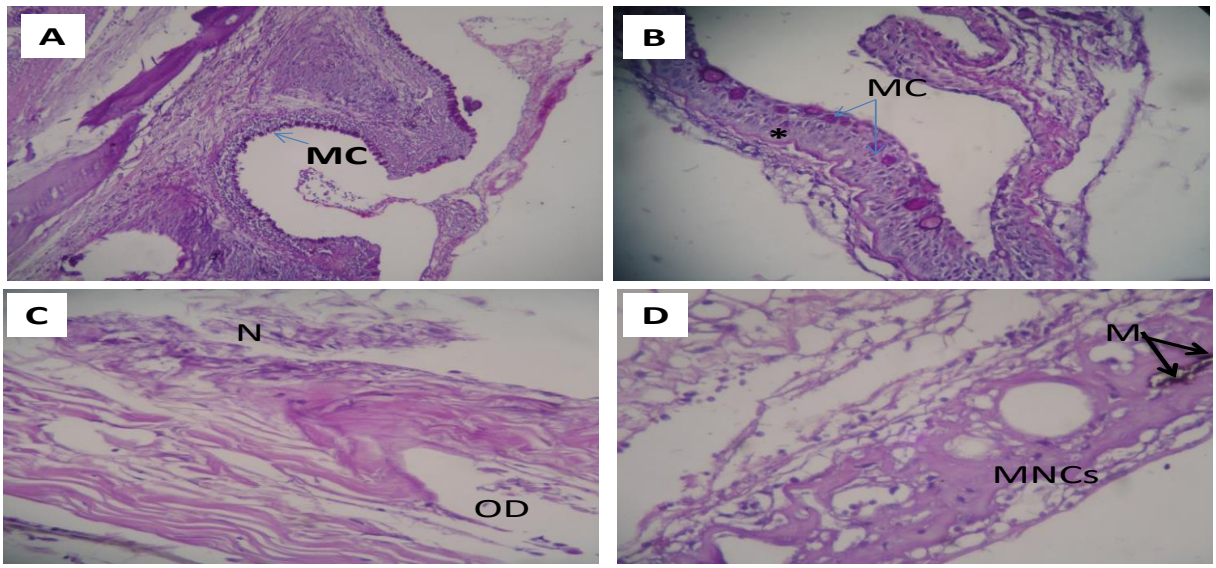

Figure 4. Photomicrograph sections showing histological structures through skin of *C. carpio* infected with *Saprolegnia spp.* and treated with Ozone **(A)** T2 showing increase number of mucous secreting cells (MC) 10x; (**B**) T3 exhibiting organized structure (*) with increased number of mucous cells (MC); (**C&D**) T4 showing dermal necrosis with intramuscular edema (OD), increase number of melanophores (M) and mononuclear cells infiltration (MNCs). PAS stain; Thickness 5-7µm. 400x.

DISCUSSION

Isolation and Identification of *Saprolegnia spp.:* The strain isolated in our study was confirmed as *Saprolegnia sp*. depending on morphological features such as the presence asexual stages (zoosporangium, zoospores and cyst), coenocytic. hyphae and the absence of oogonia as described for this genus as by Burr and Beakes (1994), Hernández *et al.,* (2003). The results of microscopically examination showed that hyphae of *Saprolegnia spp.* were clearly appeared of branched non-septet, clear and have cell membrane. All the family Saproleginiaceae characterize in this feature are in line with Coker (1923) While, the appearance of zoosporangia cylindrically or spherical in shape have many numbers of spores which is renewed by Saproleginoid this feature identified the genus of *Saprolegnia* this is similar with Seymour (1970).

Clinical Signs and Survival rate: The main clinical sings on infected group after 24-72 hr. from infection with fungal were appearance of a superficial filamentous strands called hyphae, which may extend over the body surface. These findings are in agreement with Muhsin (1977), Richards and Pickering (1978). Also, the results are in line with Seymour, (1970) who supposed that up to 40 or 50 % of the body surface and gills may be covered with hyphae. In early infections, skin lesions are grey or white in color, with a characteristic circular or crescent shape, which can develop rapidly and cause destruction of the epidermis this result is in agreement with Bruno and Wood (1994). As infection develops, lethargy and loss of equilibrium follow. The actual cause of death is likely to be associated with impaired osmo- regulation. Robert *et al.,* (2003) explained that the fungal growth in water mold is characterized by cottony, brownish spots on the body surface including the gills.

Studies have shown the ability and efficiency of ozone to eliminate pathogenic fungi, with high efficacy and without side effects (Forneris *et al.,* 2003). In this study, all treatment groups (T1, T2 and T3) were responded against the Saprolegniasis compared to infected fish (C+), particularly the T2 group (0.50 mg /l), it showed highest survival rate and increases the efficiency and vitality of the ozone in minimizing its pathogenic effects and this is in line with many studies that showed ozone ability to reduce infection, through the mechanisms referred to Hansler (2003), Calunga *et al.,* (2005) and Huth *et al.,* (2007)**.** Ozone effective in reducing Saprolegniasis in hatcheries (Forneris *et al.,* 2003) reported treatment with ozone increased egg hatching from 42.6 to 49.1% with a dose of ozone from 0.01 to 0.2 mg/l.

The clinical signs of (T3) were marked by significant changes in fish behavior due to high concentrations of ozone. Fish were stopped feeding and collected near the surface of the water, sometimes trying to pull the air out of the surface with irregular swimming and increasing attempts to jump out of the ponds. Pryor *et al.,* (1991) described the clinical signs of irradiated trout that are exposed to high concentrations of ozone, and the fish that reach this condition rarely survive.

Formalin treated group (T4) was clearly responded to treatment against Saprolegniasis in infected fish, these results are in agreement with Rabee (1992) Generally, Formalin inactivates microorganisms by alkylating the sulfhydral and amino groups of proteins and ring nitrogen atoms of purine bases 376. Although, formalin effectively used kill external parasite on skin, gill and fin but not preferred treatment for external bacterial and fungal infections due to firstly carcinogenic and tetragenic effects (Wael and Ahmed, 2013), secondly; formalin chemically removes dissolved oxygen which is conducive to development uncontrollable oxygen depletion (Fitzpatrick *et al.,* 1995).

Histopathological Studies: The main histopathological changes observed after 14 days from infection with *Saprolegnia sp*. in gills sections was in C+ group, including: epithelial lifting, hyperplasia of epithelial cells, interstitial edema, with blood congestion and necrosis. Histopathological assessment exhibited increase levels of gill epithelial proliferation and epithelial lifting, also fusion of secondary and primary lamellae in fish from ozonated systems and formalin treated group relative to C+ and C- groups. The epithelial proliferation and epithelial lifting and fusion of secondary and primary lamellae in fish are considered protective function (i.e., defense mechanism) and its unspecific responses possibly resulted by several infectious and environmental stress, such as exposure to increased waterborne heavy metal (Sutherland and Meyer, 2007).

Generally, these changes are an attempt to increase the distance between blood and external environment for oxygen and ionic exchange (Ferguson, 1989). As gill tissue consist of the largest surface area of the fish in direct contact with the surrounding environment (Evans *et al.,* 2005). In the current study, it noted gradual changes of the gill epithelium with increasing ozone levels and time of exposure. According to Mallatt (1985), the changes, (i.e., hyperplasic and hyper-

trophic alterations), can be regarded as adaptive response since these alterations increase the distance between the surrounding environment and the blood vessels and therefore protect the organism to reduce uptake of the toxicant. In contrast, necrosis represents a direct and detrimental effect of an irritant, which could be reflect a severe destruction of the gill lamellae, thereby affecting the gills functionality (Temmink *et al.*, 1983; Mallatt, 1985). Such findings are in line with observations obtained by Good *et al.*, (2011) who stated increase levels of gill epithelial hypertrophy and proliferation in ozonated water of rainbow trout, *Oncorhynchus mykiss.*

Histopathological changes in skin and muscles of *C. carpio* probably represented the increase of enzymatic activity due to *Saprolegnia*. infection. Peduzzi and Bizzozero (1977) proved that the thalli of pathogenic strains of *Saprolegnia* show chymotrypsin-like activity and proved that this enzymatic activity is possible a contributing factor to the pathogenesis of Saprolegniasis. Also, Fregeneda-Grandes (2000) have also established that these alterations in skin due to proteolytic enzymes secreting by *Saprolegnia spp*. The increased number of mucous secreting cells in Ozone treated groups possibly reflected accelerated release of mucous cell contents as a defense response. Stimulation of mucus secretion is a classic stress response in fish and has been reported earlier for other stressors such as crude petroleum, heavy metals including lead, mercury, copper and chromium (Iger *et al.*, 1994). The results of this study are in accordance with observations obtained by Amin *et al.*, (1985) and Ferguson (1989). Similar pattern of changes in the skin of Saprolegnia infected fish have been also described by Hatai Hoshiai, (1994), Hussian *et al.*, (2013) and Chauhan *et al.*, (2014). It is well known that skin act as protective barrier against numerous infectious agent. Although, some of causative agent are dermatological manifestations of systemic infections, most of them exclusively target the body surface (Noga, 2000). The specific events leading to the development of an infected damage are still unclear, but an increasingly large body of evidence shows that many non-infectious stressors can damage skin (Iger *et al.*, 1995).

In conclusion, Ozone could be used as potential and promising alternatives to chemotherapeutics compounds in aquaculture, to achieve sustainable, economic and safer fish production. Furthermore, research is required to find out the more control measures against *Saprolegnia spp.*

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Conclusion

the results indicated the effectiveness of ozone as antifungal in controlling Saprolegniasis. Thus, Ozone could be used as potential and promising alternatives to chemotherapeutics compounds in aquaculture, to achieve sustainable, economic, safer and eco-friendly fish production.

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