Evaluation the Ozone Treatment To Control the Infection of Saprolegniasis in Cyprinus carpio L.

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Abstract . The objective of present study is to set up an alternative method of controlling of saprolegniasis, in Cyprinus carpio L. using ozone. For this purpose, six different treatments were applied of 120 fish (20 fish/treatment), of which three infected with Saprolegnia spp. (2×10^4 zoospors/l) and were treated with ozone (O_3) in concentrations of 0.25, 0.50 and 0.75 mg l\(^{-1}\) (1 hour), one reference treatment with formalin (0.15 ml l\(^{-1}\), 30 min), two treatments one as positive control (infected with Saprolegnia spp. without disinfectants) and one as negative control without any disinfectants. After 14 days of treatment with ozone and formalin, blood samples were collected from fish for hematological, biochemical and Immunological tests. Survival rate was also studied. Considerable changes have been recorded in the mean values of blood indices. RBCs count and Hemoglobin content, were found significantly increased (p<0.05) in all ozone treatments compared to C- group. Total protein and globulin were significantly increased (p<0.05) in all ozone and formalin treatments in comparison to positive and negative groups (C- and C+). Albumin showed a significant decrease (p<0.05) in all ozone and formalin treatments relative to C+ and C- group. The results of respiratory burst activity (measured by Nitro blue tetrazolium reduction assay) showed significantly different (p<0.05) in all ozone and formalin treatments respectively compared to control groups (C+ and C-); Highest respiratory activity was observed in ozone treatment at 0.50 mg/l. Among Ozone treatments 0.50 mg l\(^{-1}\) showed the highest survival rate (90%) followed by formalin treatment (80%). Survival rate of the control group (without disinfectant) was 20% and there was a significant difference (P<0.05) with other treatments. It was obvious that the obtained results were highly suggestive for the efficiency of ozone as an efficient antifungal disinfectant for fish.

Keywords: Cyprinus carpio, Formaldehyde, Ozone, Saprolegnia spp., Nitroblue tetrazolium reduction assay.

1 . Introduction

Fungal infections are one of the most identified diseases responsible for highest mortality and economic losses among the fish farming productions and also can extensively destroy the fish eggs in hatcheries (Torto-Alalibo et al., 2005; Jalilpoor et al., 2006). The most important recognized fungal pathogens of fish are Saprolegniasis (water mold) of the Saprolegnia genus (Osman et al., 2008).
Infection with *Saprolegnia spp.* are widespread occurred at low temperature at any stage of fish life cycle (Hussein *et al.* 2001). Saprolegniosis characterized by a relatively cottony/woolly, white fungal growth over the skin, gills, or on fish eggs when in water. (Copland and Willoughby 1982; Whisler 1997).

Routine application of antifungal is a commonly used for controlling *Saprolegniasis* but, most of them have been considered obsolete. Previously, malachite green, formalin were the most potent fungicide for fish (Khomvilai *et al.*, 2005). Although, the use of these chemicals are effective in controlling fungal diseases, these antifungal agent are not readily biodegradable and tend to residue in the environment, chemicals and other substances that could result environmental threat (Khoo, 2000). Niska *et al.* (2009) have confirmed that malachite green is carcinogenic, mutagenic (Niska *et al.*, 2009). Thus, it has bun in many countries. Therefore, an alternative antifungal are required to be effective and safe. Among those ozone therapy has high effectiveness in cleansing, disinfection, reduction pathogens and scaled the heavy losses in fish farms through the activities of the immune system (Bullock *et al.*, 1997; Bocci, 2005). Hence, the aim of the present study is to set up an alternative method of controlling of *saprolegniasis*, in *C. carpio* L. using ozone.

**Materials and Methods:**

**Isolation and Identification of *Saprolegnia spp.* from Infected Fish**

A total number of 50 infected *C. carpio* that showed external cotton wool like fungal mat lesion and ulcerations on the pectoral, caudal fins or skin were collected from local cages in Babel/Iraq. These fish were transfered to the laboratory in sterilized polyethylene bags for further examination. The infected areas were transferred to a sterile plates of Sabouraud dextrose agar with chloramphenicol and sterile sesame seeds (*Sesamum indicum*). Culture plates were kept for 3-5 days at 20ºC in incubator with regular examination daily for any expected growth. Harvested fungal growths were purified then slide culture method was done on colonies for initial morphological identification. Fungal spores were fixed with one drop of methyl alcohol and stained with lacto-phenol cotton blue as described by Willoughby (1994). 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).

**Experimental Design of Artificial Infection**

Approximately of 120 healthy *C. carpio* with weighing 100±5g, were obtained from a commercial fish farm of Babel/Iraq. Fishes were acclimatized for two weeks in laboratory conditions before the beginning of the research. The fishes were randomly stocked into 12 plastic tanks (10 fish/tank) and subdivided into 6 treatment groups in duplicate (2 tanks/treatment). The fungi isolates were then introduced to the fish environment (2 X 10^7 zoospores per liter) and left in the aquarium tanks for 14 days. When signs of cotton wool like fungal were evident on the fish, varying concentrations of ozone were introduced into the various tank and 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 hour respectively; T4: fish were infected with *Saprolegnia spp.* and treated with formalin (as a reference) 0.15 ml/ l for 30 min for 3 successive days *Saprolegnia spp.*. Both infected and controlled groups were monitored for 4 days. Water quality parameters were monitored every day in each tank as follows:[Dissolved O2 (mg l^-1) 6.10±0.5; Temperature (°C) 22 ± 1; pH 7.10 ± 0.05] using YSI D.O. meter. After 14 days of treatment with ozone and formalin, blood samples were collected from fish for hematological, biochemical and Immunological tests. Percentage survival was also calculated as follows:

\[
\text{Percentage Survival rate} = \frac{\text{final number of fish survivor}}{\text{initial number of fish stocked}} \times 100
\]

**Blood and Serum Collection**

Blood samples were drawn from caudal peduncle. Two sets of test tubes were used the first part of blood was transferred to heparin anticoagulant tubes for RBCs (10^6/µl) and WBCs (10^3/µl) count according to method described by Hrubc and Smith (2000). Hemoglobin content (g/dl) was detected
using hemoglobin test kit. Respiratory Burst Activity was determined using the reduction of nitroblue tetrazolium (NBT assay) to formazan as a measure of superoxide anion (O$_2^-$) production as described by Mustafa (2012). The second part of blood were transferred to tubes without heparin containing gelatin and permitted to clot for 2 hr. Serum was separated by centrifugation and stored at -20°C and were used for biochemical parameters (Total proteins, Globulin and Albumin) according to method of.

**Statistical analysis**

Statistical analysis was achieved using SPSS V. 16 Software. One was analysis of variance (ANOVA) was used to determine the significant differences between variables. The differences of means were analysed at 5% probability value. A probability level equal or less than $P < 0.05$ were considered significantly different.

**Results**

**Isolation and Identification of* Saprolegnia* spp.**

**Macroscopically and Microscopically**

At first lesions are appeared as small, circular, and depigmented areas, sometimes with hemorrhagic borders. In progressive stages lesions can become ulcerative and the fish can be almost entirely covered with thick fungal growth. The morphological features of the fungal growth colonies on SDA are appeared after 24-72 h from incubation at 20°C as circular mass of filaments, whitish in color and brownish in the center and characterized by an widespread and dense mycelium. Fungus isolates were characterized by presence of branched non-septate hyphae, together with masses, various in length and width, transparent and has cell membrane. Such sporangia were filled with large number of spores which separated from the basal somatic hyphae by process called Saprolegnoid (Figure 1).

**Hematological parameters**

Results of hematological parameters are presented in Table 1. RBCs count (Cell×10$^6$/μl) recorded significant decrease ($p < 0.05$) in C+ group after 14 days from infection by *Saprolegnia* spp., compared to C- group. The highest value was registered in T2 which showed significant ($p < 0.05$) increase in comparison to all treatment groups (T1, T3, T4) and control groups (C- and C+). No significant differences were detected between treatment groups respectively and C- group.

![Figure 1: A- Many fishes were found dead in the aquaria due to the heavy invasion of the *Saprolegnia*. B- Gross lesion in common carp characterized by cotton wool like hyphal mats and ulcerations on the body. C&D- Wet culture of fully developed *Saprolegnia* spp. on sesame seeds.](image-url)
WBCs count (Cell/μLx10^3) in blood of *C. carpio* infected with *Saprolegnia spp.* (C+) registered significant increase compared to healthy fish (C-). The highest value were recorded in T4 which was significantly increase (p<0.05) relative to T1, T2 T3 and to C- group. Furthermore, there were no significant differences between C+ and T1. Also, no significant difference among the treatment groups (T1, T2 and T3) were found.

PCV (%) value registered significant decrease (p<0.05) in C+ group compared to C- group. At the end of the experimental period (i.e. 14 days) PCV (%) showed significant decrease (p< 0.05) in all treatment groups (T1, T2 T3 and T4) respectively compared to control groups (C- and C+). Also, there were significant differences (p>0.05) observed among treatment groups (T1,T2) and (T3,T4).

Hb (g/dl) content recorded significant decrease (p<0.05) between C+ in comparison with the C-group. In addition, all treatment groups (T1, T2, T3 and T4) showed considable differences (p<0.05) compared to C- and C+ groups. No significant differences (P>0.05) among treatment groups (T2,T3 and T4). Also, there was no significant differences between C+ and T1.

**Table 1:** Hematological parameters (mean± SE) of *C. carpio* which infected by Saprolegniiasis and treated with ozone and formalin.

| Groups                  | RBC cellsx10^6/µl | WBC cellsx10^3/µl | PCV(%)  | Hb g/dl |
|-------------------------|-------------------|-------------------|---------|---------|
| C-                      | 2.22±66.17        | 19.57±0.08        | 34.42±0.73 | 7.41±0.24 |
| C+ (14 days)            | 1.80±64.59        | 24.63±0.08        | 16.64±0.64 | 5.61±0.33 |
| T1 (14 days)            | 1.81±64.83        | 25.94±8.85        | 19.42±1.18 | 6.02±0.34 |
| T2 (14 days)            | 2.43±53.70        | 26.38±3.66        | 20.38±0.85 | 8.75±0.36 |
| T3 (14 days)            | 2.17±52.53        | 26.14±3.80        | 23.75±0.45 | 9.35±0.48 |
| T4 (14 days)            | 2.07±46.94        | 30.26±5.09        | 23.27±0.34 | 8.89±0.42 |

Means: SE with different alphabetic in the same column are significantly different (p<0.05) n=6.

**Biochemical Profile**

**Total Protein, Albumin and Globulin**

Results of total protein (TP), albumin (AL) and globulin (GL) in serum of *C. carpio* infected with *Saprolegnia spp.* and treated with ozone are summerized in Table 2. TP and AL levels showed significant increase (p<0.05) in C- group after 14 days over the C+ group. TP Level revealed significant increase in T1 T2 and T4 respectively compared to C- and C+ groups. However, TP level showed no significant differences (p<0.05) between T3 in comparison to C- group. While, AL level showed significant increase (p<0.05) in C+ group compared to all ozonated and formalin groups (T1, T2, T3 and T4). On the other hand, GL levels showed significant increase in C+ group after 14 days compared to C- group. Also, GL all in ozonated and formalin groups GL revealed significant differences compared to C- group. Also, GL levels showed significant differences (p<0.05) among treatment groups (T1, T2, T3 and T4).
Table 2: Total protein, albumin and globulin levels (M± S.E.) of *C. carpio* which infected with Saprolegniosis and treated by Ozone and formalin.

| Groups                  | Total Protein (g/dl) | Albumin (g/dl) | Globulin (g/dl) |
|-------------------------|----------------------|----------------|-----------------|
| Control (C-)            | 4.40 ± 0.04 A        | 1.732 ± 0.006 A| 2.07 ± 0.004 E  |
| (C+) Infected 14 days   | 3.18 ± 0.02 D        | 1.342 ± 0.002 B| 2.62 ± 0.006 C  |
| (T1) Infected 14 days   | 3.57 ± 0.03 C        | 1.108 ± 0.0014 E| 2.44 ± 0.001 D  |
| (T2) Infected 14 days   | 3.64 ± 0.01 C        | 1.133 ± 0.001 D| 2.69 ± 0.002 C  |
| (T3) Infected 14 days   | 4.35 ± 0.06 A        | 1.131 ± 0.004 D| 3.27 ± 0.00 C   |
| (T4) Infected 14 days   | 4.04 ± 0.02 B        | 1.146 ± 0.005 C| 2.90 ± 0.001 B  |

Means± SE with different alphabetic letters in the same column are significantly different (p<0.05). n=6

**Immunological test**

**Nitroblue tetrazolium (NBT) reduction assay**

The respiratory burst activity (NBT reduction) of neutrophils of *C. carpio* of the experimental groups is shown in Figure 2. At the end of experimental period (i.e. 14 days) C+ group showed significant differences (p<0.05) in comparison to C- group. Also, NBT of all ozonated and formalin groups were significantly different (p<0.05) over control groups (C+ and C-). The highest respiratory activity was found in T2 which was significant increase compared to T1 and to T3. However, there were no significant differences (p>0.05) in respiratory burst activity between T2 and T4 in percentage.

![Figure 2](image.png)

*Figure 2:* Respiratory burst activity (NBT reduction) of neutrophils of *C. carpio* infected with *Saprolegnia* spp. and treated with ozone and with formalin. Different alphabetic letters indicated significantly different (p<0.05) Values are Means± SE; n=6.
Percentage Survival Rate

Among Ozone treatments 0.50 mg l⁻¹ showed the highest survival rate (90%) followed by formalin treatment (80%). Survival rate of the control group (without disinfectant) was 20% and there was a significant difference (P<0.05) with ozonated and formalin treatments (T1, T2, T3 and T4) (Figure 3).

![Figure 3: Survival rate of C. carpio after 14 days from the infection with Saprolegnia spp. and treated with ozone and formalin. Different alphabetic letters indicated significantly different (p<0.05).](image)

Discussion

Isolation and Identification of Saprolegnia spp.

The water molds are well known in freshwater and represent the most important fungal group affecting wild and cultured fish and their eggs, all isolates from infected fish have appeared as circular colonies after 48h incubation in SDA. These findings are in line with Bruno and Stamps (1987) also are in agreement with Muhsin, (1989) who use sesame seed for growth of Saprolegnia spp. as an alternative of Cannabis sativa L. that used by Coker (1923) and Seymoure (1970).

Hematological parameters

In the present study, the increases in WBC count in infected C. carpio (C+) could be due to a response of cellular immune response to fungal infection and with an increase in antibody production which helps in survival and recovery of the fishes exposed to the infection (Seth and Saxena, 2003). This are in line with Shah et al. (2015) who explained that the increases in WBC count in infected samples were accepted as a response of cellular immune system to fungal infection. The results showed an increase in the number of white blood cells at 0.25 and 0.50 mg /l of ozone, while high concentrations (0.75 mg/l) had a inhibitory effect resulting in a decline in the WBC count. This is consistent with Bocci (1999), who observed that ozone is a substance that stimulates the cellular immune response in general at low concentrations, while its high concentrations have a inhibitory effect. The effect of dissolved ozone in water at low and medium concentrations is due to its increased production of cytokines such as interferon factor and interleukins, particularly IL-2, which play an important role in increasing the differentiation, stimulation and regulation of lymphocytes (T cell and B cell), in disease resistance and decrease the invading germs to the body and
reduce their effects in the infected tissues and this is in line with Null (1995) and Yamaguchi et al. (2011), which explained to an increase in the number of T-lymphocytes, when exposure to safe concentrations of ozone, while high concentrations of it may be hopeless by their amount. The elevation of WBC count in formalin-treated group (T4) in this study could be attributed to the generalized stress response leading to an increase in the pituitary internal activity with an increase in the secretion of corticosteroid (Salonius and Iwama, 1993).

The present study showed that the 0.50 mg/l of ozone caused an increase in the RBC count. This may be due to the fact that ozone stimulates the blood cycle in the body by oxidation of fat and cholesterol in the walls of blood vessels. To increase the flexibility of red blood cells and increase their efficiency to carry oxygen, resulting in an increase in the level of oxygen absorbed in living tissues, as well as its role in increasing the production of ATP, which prepares cells great energy. This is consistent with Bocci et al. (2001) and Paolo et al. (2004), who observed that ozone acts to oxidize plaques in blood vessels, which helps to remove them and then the blood and lymphatic velocity. In addition, ozone activates vasodilator (vasodilatation) and increased Prostacyclin production, which inhibits blood vessel aggregation (Pressman, 1998).

The results of the present study showed the high concentration of dissolved ozone (0.75 mg/l) caused a decrease in the RBC count. The reason is that high concentrations of ozone have an oxidative effect against some of the enzymes necessary to regulate the growth and differentiation of blood cells as well as its ability to penetrate the membranes of the blood cells causing their degradation. These results are consistent with Schulz et al. (1981) 0.70 mg /l of ozone reduced the total number of red blood cells. Some studies have indicated that RBC in fish are the primary target of excessive use of ozone and not gill tissue (Wedemeyer et al., 1979; Fukunaga et al., 1991; Fukunaga et al., 1992). On the other hand, in (T4) subject to formalin revealed a significant increase of RBCs, Hb and PCV at 14 day of the experiment. These data are in line with Jung et al. (2003) who observed an increase in the values of red blood parameters (RBC count, PCV%, Hb concentration) accompanied by an increase in erythroblast frequency in Paralichthys olivaceus following formaldehyde treatment.

The current study exhibited the real effect of dissolved ozone in its various concentrations in maintaining levels of hemoglobin concentrations compared with control treatment. This is due to the role of ozone known to provide the ideal conditions for the cell of oxygen and nutrients to carry out its natural physiological functions, which reflected positively on the health of processed fish and make them able to make the most of the content of food nutrients and minerals, especially iron and copper, this is consistent with the study of Rakovsky and Zaikov (2009).

The results of PCV% value showed significant increase in ozone treatment groups (T1,T2 and T3) over C+ group due to the role of ozone in making the environment surrounding the fish rich with oxygen, which directly effects on the external parts and internal organs of the fish, as it increases the amount of oxygen entering the cells and increases the rate of structural metabolism for its role in increasing the work of energy houses (Mitochondria), which leads to improved performance of biological processes, and thus an abundance in the production of red blood cells and the subsequent increase in cell size and plasma, and this corresponds to the study of Plessis et al. (2008) who recorded a high volume of blood cells embedded in human blood samples treated with ozone outside the body (In vitro).

**Biochemical Profile**

Results of TP and AL levels revealed significant decrease (p<0.05) in (C+) after 14days from experimental period compared to control group (C-). This result are in agreement with Shah et al. (2015) who found that total serum protein and albumin content in the Saprolegnia infected rainbow trout decreased significantly as compared to the control group this might be due to the impact of dermato/systemic mycosis. Hence, it can be concluded that stress caused by fungal infection leads to...
haemostatic imbalances in fish which is reflected in the haemato-biochemical profile of infected fish. The current findings are confirmed by the results of Mastan et al. (2009). While, results of GL level showed significant increase (p<0.05) in (C+) after 14 days compared to control group (C-) this result are similar with Bly et al. (1994). Shah et al. (2015) have revealed increase in globulin levels of fish could be attributed to activation of hormonal immune response against fungal invasion at ambient temperatures. Results of TP and GL levels revealed significant increase (p<0.05) at the end of experimental in all treatment group (T1, T2, T3 and T4) in comparison with (C-) and (C+). These results are in agreement with Johansson-Sjobeck and Larsson, (1979), Sung et al. (2008) and Shah et al. (2015) who explained that GL increase due to activation immunological function against fungal invasion as a protective response in fish when expose to the stress. This indicated that the fish starting for healing the infected area by disappearing the fungal hyphae from the surface of skin, the color of skin return to normal, reduce stress and the fish returned to normal state. On the other hand, AL level in all treatment group T1, T2, T3 and T4 showed significant (p>0.05) decrease, relative to control groups (C+, C-). This could be due to ozone and formalin which are disinfectant and all disinfectant have toxicity on fish which lead to this decrease compared to the normal group, this result are in agreement with the result of Omorogie et al. (1994) who observed GL increased in formalin-treated carp and Nile tilapia Oreochromis niloticus.

Nitroblue tetrazolium (NBT) reduction assay

The reduction of nitroblue tetrazolium (NBT) by radical oxygen produce from neutrophils cell of C. carpio in (C+) showed a significant increase in NBT reduction, this confirmed that nonspecific immunity improved in infected fishes due to enhances the immunity response to overcome the stress caused by infection. Results of current study showed significantly increase (p<0.05) in NBT reduction in C+ after 14 days compared to C- group this could be due to a response of cellular immune system to fungal infection, this are in line with Shah et al. (2015) who explained that the increases in NBT reduction in infected samples were accepted as a response of cellular immune system to fungal infection. The results showed significantly increase (p<0.05) in NBT reduction at low and medium concentrations of ozone (0.25 and 0.50 mg/l), while high concentrations (0.75 mg/l) had a inhibitory effect resulting in a decrease in the number of NBT reduction this is consistent with Bocci (1999), who noted that ozone is a substance that stimulates the cellular immune response of the immune system in general at low concentrations, while its high concentrations have a inhibitory effect. The decrease of formaldehyde-treated group (T4) level of NBT reduction in this study could be attributed to the generalized stress response leading to an increase in the pituitary internal activity with an increase in the secretion of corticosteroid (Rubenstein and Pelet 1973; Gupta and Steigerwald,1974). These changes indicated that the rise of neutropoietic activity, particularly in formaldehyde-treated fish, exposed to therapeutics this result corroborated the report of Akpoilih and Adebayo (2010). Therefore, formaldehyde is not usually recommended for treating commercial fish ponds because each 5 mg/l of formalin added to a pond chemically removes 1 mg/l of dissolved oxygen from the water (Schreier et al., 1996). Furthermore, formaldehyde is a mutagen and carcinogen even at low concentrations and presented toxicity in a variety of organisms (Srivastava et al., 2009).

Survival Rate

Ozone at 0.50 mg/l showed highest survival rate this indicating the efficiency of the ozone in reducing its pathogenic effects and this is in agreement with several 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.

In conclusion, the obtained results were highly suggestive for the efficiency of ozone as an efficient antifungal disinfectant for fish. In addation to that, Ozone is preferred to formalin and can be considered as one of the valuable disinfectants, because of a lower concentration of ozone needed to disinfect the
culture medium, therefore, environmental contamination will be decreased consequently this will reflected on human health.

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