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EVALUATION OF HYDROGEN PEROXIDE ON CONTROLLING SAPROLEGNIASIS IN COMMON CARP, *CYPRINUS CARPIO* L.

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ABSTRACT : This study was aimed to investigate the efficacy of hydrogen peroxide on controlling mortality associated with Saprolegniasis in common carp, *Cyprinus carpio* L. Saprolegniasis was experimentally induced in common carp at concentration of 2×10^4 zoospore/L. A total of 100 fish were randomly divided into five groups (fish/treatment) control negative group, abraded but not challenged, infected group, challenged with *Saprolegnia* spp. The 2nd, 3rd, 4th and 5th groups were challenged with *Saprolegnia* spp. and therapeutic hydrogen peroxide bath treatments of 13 and 6 mL/L for 30 min addition to formalin bath of 0.15ml/L for 60 min were administered respectively for 3 days interval. Hydrogen peroxide treatments of 6 mL/L were harmful (relative to lower concentrations) to test fish and resulted in 35% mortality. Fish groups treated with H_2O_2 at concentrations of 1 and 3 ml/L reported the lowest mortality rate reached up to 10, 25% respectively, followed by formalin treated group reached up to 20%. Mortality rate in untreated positive control reached up to 80%. After 15 days from the experimental period, albumin level in T_1, T_2, T_3 and T_4 registered significant increase ($p<0.05$) compared to C+C- groups. While, globulin and total protein showed significant increase in T_1 and T_4 relative to C+ group and there was no significant difference between T_2 and T_3 groups. At the end of the experimental period there was significant increase ($p<0.05$) in ALP activity in all treatment groups were over the C- and C+ groups. However, there was no significant differences ($p>0.05$) among all treatment groups. Although, the use of H_2O_2 did not completely reduce fungal infection, but it may diminish the frequency of the use of hazardous chemicals such as malachite green. However, search for alternatives therapies must continue and it is of vital importance for the development of effective treatment against Saprolegniasis in fish.

Key words : Common carp, hydrogen peroxide, *Saprolegnia* spp., fish environment, alkaline phosphatase.

INTRODUCTION

Aquaculture production has grown progressively in the last five decades with food fish supply growing yearly at 3.2% and global population increasing at rate of 1.6%, however the gap is decrease (FAO, 2014). Although, intensive aquaculture fish are exposed to several stressors that affect their physiological status and cause higher susceptibility to infectious diseases and leading tosignificant economic losses. Saprolegniosisis a major aquatic disease for global fish production. This disease cause a serious problem in fish culture. *Saprolegnia* lesions characterized by brownish areas of cottony wool-like appearance growth on the skin when the hyphal elements spread out (Durborow *et al*, 2003). Natural recovery is unlikely in most cases; thus, effective fungicides are required. Although, malachite green and formalin are effective for controlling of Saprolegniasis on fish and fish eggs, butthey banned by the Food and Drug Administration (FDA) to use as a fungicide in fish culture systems in the U.S. (Marking *et al*, 1994; Fitzpatrick *et al*, 1995). Due to teratogenic properties (Meyer and Jorgenson, 1983; Fitzpatrick *et al,* 1995) andthe residue of these chemicalesaccumulated in the environment (Meinertz *et al*, 1995).

Hydrogen peroxide has been reported to effectively control Saprolegniasis on fish and fish eggs (Marking *et al*, 1994; Schreier *et al*, 1996). Recently, hydrogen peroxide use is allowed under a ''low-regulatory priority'' ruling by the FDA in the United Statesfor controlling Saprolegniasis in fish and fish eggs. Unpublished and few studies and also hatchery field trials with hydrogen peroxide have revealed promise that H_2O_2could be effective for controlling Saprolegniasis (Schreier *et al*, 1996; Rach *et al*, 1998). Thus, definitive studies are needed to evaluate efficacy and determine ideal therapeutic treatment. Therefore, the present study,was designed to assess the efficacy of H_2O_2 treatment on biochemical, hematological andbehavior of *Cyprinus carpio* following Saprolegniasis infection.

MATERIALS AND METHODS

Saprolegnia sp. isolation and identification

Isolation and Identification of fungiwas carried out according to Ashour *et al* (2017) from water media and from naturally infected fish. Samples were collected from common carp showingmycelia growth (cottony-wool like appearance) on the skin and transferred to a sterile plates containing 15 ml of autoclaved distilled water. After that, sterilized sesame seeds, *Sesamum indicum* (5-9 seeds) were added as a bait substrate. Then, the plates and incubated at 25ºC for 72-94 hrs. Single colonized sesame seeds were aseptically transferred to autoclaved Sabouraud Dextrose agar (SDA, Difco) with adding of Chloramphenicol at concentration of 0.05 g/L to avoid the bacterial contamination and to obtain a pure culture. Identification of the isolates werecarried out based on morphological features and microscopical characteristics (Willoughby, 1985). Hyphal colonies were identified to genus and counted to obtain the mean number of zoospores/ml. Viable fungal suspension of Saprolegnia was detected and adjusted at a concentration of 2×10^4 zoospores l^{-1} using haemocytometeraccording to the method described by Horwitz *et al* (1975).

Hydrogen peroxide (test chemical)

Hydrogen peroxideat percentage of 35% (active ingredient) was purchased fromSigma-Aldaich. All treatment concentrations were prepared based on active ingredient by dissolving a calculated volume of H_2O_2 in a specific volume of water adequate to treat the fish for 30 min.

Acclimation of fish and experimental setup

A total number of One hundred male *C. carpio* (common carp); weight 50 ± 2.0 g, length 15±1.5 cm were held in ten glass aquaria (n = 10 fish/aquarium), with 70 L of water. Continuous aeration was maintained in each aquarium using an electric air pump, pH was maintained at 7.5±0.3, temperature was maintained at 23.2±1.2ºC, dissolved oxygen at 7.3±1.0 mg/L, ammonia at0.003±0.01 mg/L, nitriteat 22.10 ± 0.05 mg/L and a12:12-h light: dark photoperiod. Fish were fed with commercial fish diet at a rate of 2% of body mass during the experiment. Fishes were acclimated for 15 days before the starting of the experiment.

After two weeks of acclimatization, fish were randomly classified into 5 groups: control and fourth treated groups. Each group consisted of twenty fish (10 fish/tank in duplicate). The first group was served as a control (10 fish served as negative control (C-) and 10 fish served as positive control (C+). The second, third and fourth groups (T_1, T_2 and T_3) were infected systematically with *Saprolegnia* spp. at concentration of 2×10^4 zoospore/L and treated with 1, 3 and 6 ml/L respectively hydrogen peroxide for 30 min. The fifth group (T_4) was infected with *Saprolegnia* sp. at concentration of 2×10^4 zoospore/L and treated with formalin (0.15 ml/L) for 60 min. The duration of treatment was three successive days interval. A total of six fish from each treatment group wereused for collectingblood samplesby pooling at the 7th and 15th days of the experiment. Blood samples were collected from the caudal vessels andleft to clot and then centrifuged at 3000 rpm for separation of serum for biochemical profile.

Clinical sings and survival rate

Abnormalities in fish behavior, clinical sings and mortalities of all treatment groups were registered during the experimental period (*i.e.* 15 days).

Biochemical profile

The serum alkaline phosphatase (ALP) activity was measured by using diagnostic kits (Bayer Diagnostics, Baroda, India). The total protein was determined as described by Grant *et al* (1987). The serum albumin level was measured according to the method of Doumas *et al* (1981). The serum globulin was estimated by subtracting the albumin from the obtained total protein as described by Doumas and Biggs (1972).

Statistical analysis

Statistical analysis was achieved using SPSS Inc., Chicago, IL, version 20.All results were expressed as mean ± SER. Comparison among groups was done using a one-way and two- way analysis of variance (ANOVA). A probability level equal or less than 5% ($P < 0.05$) was considered significantly different.

RESULTS

Mycological examination

Results of fungal examination showed that the positive colonies on (SDA) at 25ºC for 48-72 hrs started with growths of long hairs with white- cottony colorafter that became grey then black after 4 days of incubation The wet samples of body surface and mouth lesions revealed masses of immature and mature sporangia filled with large number of sporangiospores, the hyphae appeared abundantly branched and were non-septated, these morphological observations were characteristic of the *Saprolegnia* species (Fig. 1 A & B).

Clinical sings and mortality rate

The affected fish had typical clinical signs of water mold infection, with cotton-wool like masses on the mouth and head and also appearance on tail, dorsal and pectoral fins. Also, the infected fish showed erratic swimming,

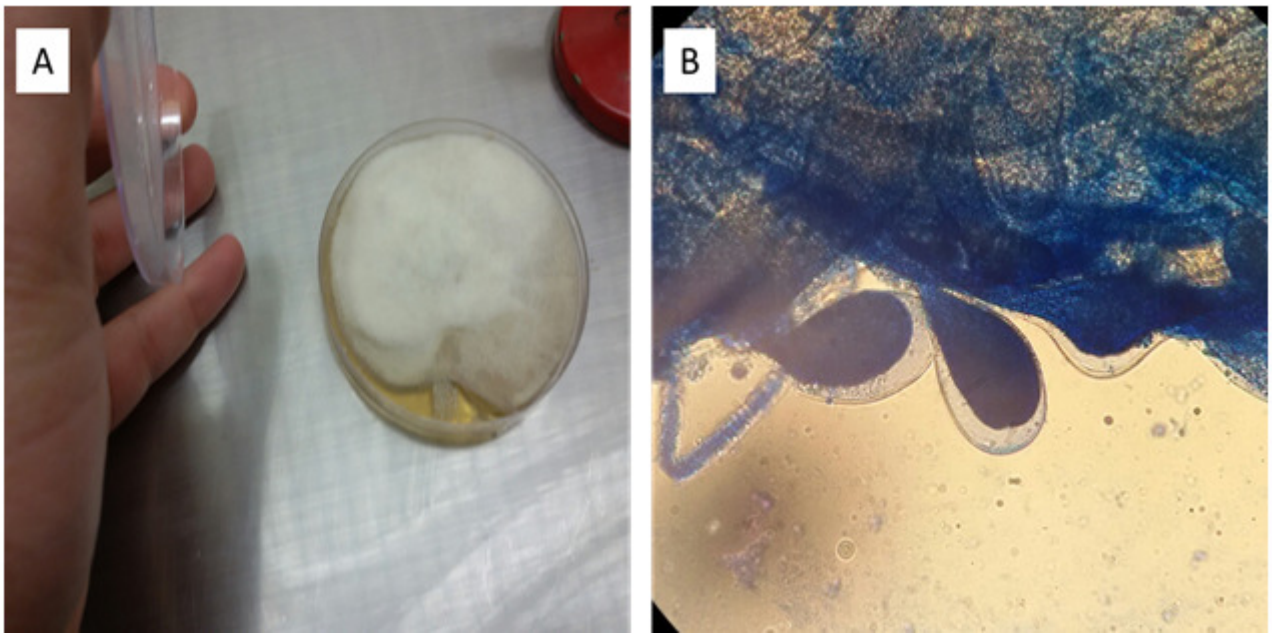

Fig. 1 : A. The positive colonies on SDA at 25ºC for 72-94 hrs started with growths of long hairs with white color and cottony- wool appearance after 94 hrs became grey then black in color. **B.** The wet specimen of skin lesions showed masses of immature and mature sporangia filled with large number of sporangiospores, the hyphae appeared abundantly branched and were non septated.

Fig. 1 : *C. carpio* experimentally infected with 2×10^4 of *Saprolegnia* spp. showing cotton wool like growths on the body surface.

listlessness, loss of appetite and rising near water surface or resting with their abdomen on the aquarium. All dead fish showed mycelia growth on the body surface (Fig. 1). The H_2O_2 gave a good influence in the treatment and disappearance of fungal growth and clinical signs in affected common carp in aquaria especially in a concentration of 1and 3 ml/Lfor 30 minutes for 3 successive days.

Mortality rate in common carp was increased simultaneously with the H_2O_2 concentration and reached up to 35% in the group exposed to 6 ml/L H_2O_2. However, Fish groups treated with H_2O_2 at concentrations of 1and 3 ml/L reported the lowest mortality rate reached to 10, 25% respectively, followed by formalin treated group mortality rate reached up to 20%. In contrast, Fungal infection in the positive control group was extremely high, at mortality rate reached to 80%. No mortality was recorded in any fish of the control negative group (Table 1).

Biochemical profile

Albumin, globulin, total protein and alkaline phosphatase

Results of albumin, globulin, total; protein and alkaline phosphatasein serum of *C. carpio* after 7 and 15 days from infection with *Saprolegnia spp.* are summarized in Tables 2 and 3. After 7 days from the experimental period Albumin level showed significant decrease in T_1, T_3 and T_3 compared to C+ group. However, T_4 recorded significant increase ($P<0.05$) compared to all H_2O_2 groups.

On the other hand, TP and globulin levels showed significant increase ($p<0.05$) in T_1 and T_2 compared to C+. Also, TP and globulin of T_1 and T_2 registered significant increase in comparison to T_3 and T_4. ALP activity showed significant decrease ($p<0.05$) in all treatment groups (T_1, T_2, T_3 and T_4) compared to C+ group).

After 15 days from the experimental period, albumin level in T_1, T_2, T_3 and T_4 registered significant increase ($p<0.05$) compared to C+C- groups. While, globulin and total protein showed significant increase in T_1 and T_4 relative to C+ group and there was no significant difference between T_2 and T_3 groups. At the end of the

Table 1 : Common carp, *C. carpio* mortalities per week, total mortalities (TM) % and survival % out of 20 fish /group for 2 weeks (1st W and 2nd W) to 2×10^4 zoospores/L of *Saprolegnia* spp.

Treatment groups	Mortality		TM%/Group	Survival%
	1st W	2nd W		
C-	-	-	-	100
C+	7	9	80	20
T_1	-	2	10	90
T_2	3	2	25	75
T_3	5	2	35	65
T_4	3	1	20	80

C- negative control; C+ positive control; T_1, T_2 and T_3 fish infected with *Saprolegnia spp.* and treated with H_2O_2 at concentrations of 1, 3 and 6 mg/L respectively; T_4 fish infected with *Saprolegnia* spp. and treated with formalin at concentration of 0.15 ml/L.

1996). Our results clearly revealed the pathogenicity of Saprolegnia to common carp associated with higher mortalities. The current results were in line with Zahran and Risha (2013), who reported that the cumulative mortalities in challenged Nile tilapia, *Oreochromis niloticus* exposed to 2×10^4 spore/L concentration of *Saprolegnia ferax* were 30% in control positive group. The present result are also in agreement with Ashour (2017), Hamed and Mustafa (2018) whom found that the accumulative mortalities in *C. carpio* exposed to 2×10^4 spore/L concentration of *Saprolegnia* spp. were 50% and 80%, respectively.

Furthermore, Hussein and Hatai (2002) found that the cumulative mortalities of the different salmonids fish groups exposed to 2×10^5 spore/L concentrations of *S.*

Table 2 : Total protein, albumin and globulin (g/dl) and ALP (U/L), (M± S.E.) of *C. carpio* after 7 days from infection with *Saprolegnia sp.* and treated with H_2O_2.

Groups	Albumin g/dl	Globulin g/dl	Total protein g/dl	ALPU/L
C-	1.89 ±0.06b	3.51±0.03a	5.41±0.10a	46.40±7.21c
C+	1.97±0.04a	2.56±0.03b	4.53±0.09b	153.2±9.32a
T_1	1.81±0.09c	3.48±0.08a	5.28±0.09a	42.4±3.04cb
T_2	1.80±0.01c	3.38±0.01a	5.28±0.08a	57.04±5.34b
T_3	1.81±0.04c	2.67±0.07c	4.49±0.07b	49.27±8.10c
T_4	2.05±0.08a	2.95±0.03c	4.99±0.04b	35.41±5.22d

Values with different small letters in the same column are significantly different ($p<0.05$), n=6.C- negative control; C+ positive control; T1, T2 and T3 fish infected with *Saprolegnia spp.* and treated with H_2O_2 at concentrations of1,3 and 6 mg/L respectively; T4 fish infected with *Saprolegnia* spp. and treated with formalin at concentration of 0.15 ml/L.

Table 3 : Total protein, albumin and globulin (g/dl) and ALP (U/L), (M± S.E.) of *C. carpio* after 15 days from infection with *Saprolegnia sp.* and treatment with H_2O_2.

Groups	Albumin g/dl	Globulin g/dl	Total protein g/dl	ALPU/L
C-	1.89±0.04b	3.51±0.03c	5.41±0.02c	46.40±7.21c
C+	1.91±0.02b	3.97±0.07b	5.88±0.02b	52.25±3.65b
T_1	2.02±0.05a	4.46±0.09a	6.36±0.08a	65.00±4.54a
T_2	2.01±0.05a	3.87±0.07b	5.88±0.01b	59.00±7.32a
T_3	2.13±0.01a	3.77±0.02b	5.905±0.04b	66.00±4.50a
T_4	2.08±0.01a	4.55±0.02a	6.64±0.01a	61.50±5.04a

Values with different small letters in the same column are significantly different ($p<0.05$), n = 6.

experimental period there was significant increase ($p<0.05$) in ALP activity in all treatment groups were over the C- and C+ groups. However, there was no significant differences ($p>0.05$)among all treatment groups.

DISCUSSION

Saprolegniosis is one of the most important oomycetes infectionsthat can causesignificant economic losses in culturedand wild fish (Van West, 2006; Phillips *et al*, 2008). The death of fish mainly occurred due to the impaired osmoregulation caused by the damaged epidermis over a larged surface area (Bruno and Poppe,

salmonis were 100% for rainbow trout, 93.3% for sockeye salmon and 90% for brown trout. Yanong (2003) demonstrated that the Saprolegnia lesions were in form of cottony wool like growth on different parts on fish body surface primarily in the dorsal region and on the dorsal and caudal fins which in same event with our resultsin which the lesion appeared mostly in all sites of the body surface.

Both H_2O_2 treatments of 1 and 3 ml/L obviously caused some treatment- related mortality reached up to 10 and 25%, respectively. However, the mortality rate atconcentration of 6 ml/L H_2O_2 was 35%. This could be

due to an additional chemical stress to the fish.

Howe *et al* (1999) recommended that the optimum hydrogen peroxide concentration for preventing or controlling mortality, reducing the incidence of infections, and enhancing the recovery of infected channel catfish with Saperolegnosis was 7.5 ml H_2O_2/L. All treatment groups (T_1, T_2 and T_3), which treated against the Saprolegniasis revealed recovery sings compared to infected fish (C2) this indicated that H_2O_2 was very active disinfectant can kill and depressing the fungal.

Formalin treated group (T_4) was clearly responded to treatment against Saprolegnia infected fish, this finding is in corresponded with result of Rabee (1992), who showed the effectives of formalin for controlling water mold disease in fish and fish eggs. Although, formalin is effectively used to kill external parasite on skin, fins and gills however are not preferred treatment for infections microorganisms due to carcinogenic effects (David, 2012) and formalin chemically removes dissolved oxygen which is contributing to development of hypoxic condition. Besides, large amount of formalin are required to treat even a small farm. As well, the highest cost and risk associated with use of formalin in fish farms make its used in ponds difficult to validate (Fitzpatrick *et al*, 1995).

Biochemical analysis, exhibited variation in level of Albumin, total protein, globulin Albumin level after 7 days from the experiment revealed a moderate increase in the infected group relatedto the control negative group. Also, the C+ group showed significant increase compared to H_2O_2 treatment groups. This could be happen as a consequence of epidermal damage due to Saprolegnia also, through skin ulcers present on skin surface, these can be reflected an entrance of loss of plasma protein or that excess of water influx to the body of fish resulting disruption in the electrolytes andimpaired of osmoregulation (Tripathi *et al*, 2005). However, after 15 days from the experimental period, Albumin level showed significant increase in all treatment groups relative to C+ group. This result indicated that the recovery of fish is started. This results is in line with Ashour (2017), who found increasing in albumin level in carp fish after 16 days from infection with Saprolegnia and treatment with Virkon S.

While, Globulin level showedsignificant increase after 7 days in H_2O_2 groups in comparison to C+ group. Our results were in accordance with Shah *et al* (2015) and Ashour (2017) whom pointed the increase in globulin level of fish might be due to stimulation of humoral immune response against Saprolegnia infection.

After 7 and 15 days from the experimental period, the plasma protein level showed significant decreased in H_2O_2 treated groups compared to C+ group. This result is corresponded with Biswas *et al* (2006), who found reduction in serum protein levels inred sea bream Pagrus major after acute handling stress and in common carp following quarantine (Ruane *et al*, 2002).

The result of ALP showed marked increasein all H_2O_2 treated groups after 15 days from the experimental period All these results indicate that H_2O_2 increases the resistance of *C. carpio* so that it can withstand the adverse conditions of infection. This result is corresponding with Rao *et al* (2006), who found enhanced in ALP in *Labeo rohita* infected with *Aeromonas hydrophila* and treated with *Achyranthes aspera*.

CONCLUSION

The present study found that 1 and 3 ml/L H_2O_2 were best for fungal control and with disappearance of clinical sings. Although, the use of H_2O_2 did not completely reduce fungal infection, but it may diminish the frequency of the use of hazardous chemicals such as malachite green. However, search for alternatives therapies must continue and it is of vital importance for the development of effective treatment against Saprolegniasis in fish, inspired by economic losses in the aquaculture production as well as the effect on animal welfare.

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