ASSESSMENT OF HYDROGEN PEROXIDE ON HISTOPATHOLOGY AND SURVIVAL RATE IN COMMON CARP, *CYPRINUS CARPIO* L. INFECTED WITH SAPROLEGNIASIS

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ABSTRACT

The present study was carried out in order to understand and describe the histological alterations and survival rate in common carp, *Cyprinus carpio* L. treated with various concentrations of hydrogen peroxide following Saprolegniasis infection. One hundred fish infected with *Saprolegnia spp*. were collected from fish farms in Babylon/Iraq. Fungal isolation was done from different parts of the body using the routine technique. Pathogenicity of the isolated fungi was tested on host fish with a concentration of 2×10^4 zoospores L^{-1} and the fishes were got the infection. Hydrogen peroxide at different concentrations (1, 3, 6 ml/L for 30 min for 3 successive d and control group) was used to control the experimental fungal infection in fish. In addition, formalin (0.15 ml/l for 60 min for 3 successive d interval) was used as a reference treatment. Results showed various histomorphological changes in the gills of the infected and treated fish with H_2O_2 including degeneration of lamellae, epithelial proliferation, fusion of the secondary lamellae, aneurysm and cellular necrosis. Similar alterations were seen in fish infected groups and treated with H_2O_2 or formalin but are significantly decrease ($P<0.05$) compared to positive control group. The skin of the positive control revealed loss of epidermal layer, necrotizing of dermis and hypodermis with mononuclear infiltration also were seen. Treatment group at concentration of 1mg/L H_2O_2 offered the highest survival rate (90%) followed by 3 and 6 ml/L (75 and 65%) respectively. In conclusion, the present study approved the potency of the hydrogen peroxide in controlling Saprolegniasis in common carp.

Key words: fish, fungal infection, *Saprolegnia spp*., water mold, zoospores

مجلة العلوم الزراعية العراقية -2019 :50(2):697-704 **مصطفى وآخرون**

تقييم بيروكسيد الهيدروجين في التغيرات النسجية ومعدل البقاء في اسماك الكارب الشائع المصابة بمرض عفن الماء

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المستخلص:

أجريت الدراسة الحالية لغرض وصف التغيرات النسجية ومعدل البقاء في اسماك الكارب الشائع .Cyprinus carpio L، المعاملة بتراكيز مختلفة من بيروكسيد الهيدروجين بعد الإصابة بخمج Saprolegniasis. تم استخدام مجموعة من 100 سمكة مصابة *بفطر .Saprolegnia spp* تم جمعها من مزارع الأسماك في محافظة بابل/العراق. تم العزل الفطري من أجزاء مختلفة من جسم السمكة باستخدام التقنيات المختبرية. تم أجراء اختبار الإمراضية للفطريات المعزولة على الأسماك بتركيز 2×10^4 zoospores/لتر وأصيبت الأسماك بالعدوى. تم استخدام بيروكسيد الهيدروجين بتراكيز مختلفة (1, 3 ، 6 مل / لتر لمدة 30 دقيقة لمدة 3 أيام متتالية بالإضافة الى مجموعة السيطرة السالبة والموجبة) للسيطرة على العدوى الفطرية التجريبية في الأسماك. فضلا عن، تم استخدام الفورمالين (0,15 مل / لتر لمدة 60 دقيقة لمدة 3 أيام بين يوم واخر) لغرض اختبار شدته في السيطرة على العدوى الفطرية. بينت النتائج الى وجود تغيرات نسجية مرضية متنوعة في خياشيم الأسماك المصابة والمعاملة بالبيروكسيد. تضمنت تنكس في الصفائح الثانوية وانفصال ظهارة الصفائح الثانوية مع توسع الشعيرات للصفائح الثانوية فضلا عن اندماج الصفائح الثانوية والنخر الخلوي. شوهدت تغيرات مماثلة في مجاميع الاسماك المصابة بالفطر والمعاملة باستخدام بيروكسيد الهيدروجين أو الفورمالين ولكنها كانت أقل شدة من مجموعة السيطرة الإيجابية .تضمنت التغيرات النسجية في جلد الأسماك المصابة فقدان في طبقة البشرة ولوحظ تنخر في طبقة الأدمة مع ارتشاح خلايا أحادية النواة. سجلت المجاميع المعاملة بتركيز 1 مل/لتر أعلى معدل البقاء (90٪) تليها 3 و 6 مل/لتر (75 و 65٪) على التوالي. يمكن الاستنتاج من الدراسة الحالية على فعالية بيروكسيد الهيدروجين في السيطرة على مرض Saprolegniasis في أسماك الكارب الشائع

.الكلمات المفتاحية: الشعيرات–الخمج الفطري–فطر السابرولكنيا–خياشيم–السبورات الفطرية

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INTRODUCTION

Saprolegniasis in teleost fish is a major problem affecting both wild and farmed freshwater fish productions (18, 29). But, this disease is more abundant in the cultured fish which adversely affects fish industry (14). Several predisposing factors are responsible for increasing the susceptibility of Saprolegnia infections such as malnutrition, physical injury and bad water quality (24, 27). Infections of fish by this fungi generally cause lesions of cottony/woolly, white fungal growth over the skin, gills, or on fish eggs which subsequently become enlarge and could lead to death of fish (23). Various alternative methodes have been used for contolling Saprolegniasis such as ozonated water (11), however this application is not useful in fish rearing in cages or in ponds. Antifungal agents are required to maintain healthy stocks of fish in the aquaculture systems. Hydrogen peroxide (H_2O_2) has been shown to effectively control fungi on fish and fish eggs (26, 28). Hydrogen peroxide use is recently allowed under a ''low-regulatory priority'' presiding by the Food and Drug Administration (FDA) for controlling Saprolegniasis in fish and fish eggs in the United States. Unpublished and limited data and also hatchery field trials with H_2O_2 have shown promise that it might be effective for controlling Saprolegniasis. Thus, definitive controlled investigations are required to obviously evaluate efficacy and determine ideal therapeutic treatment. The present study was carried out in order to assess the efficacy of different concentrations of H_2O_2 treatment on histopathological alterations and survival rate in common carp infected experimentally with Saprolegniasis.

MATERIALS AND METHODS

SIsolation and identification of *Saprolegnia spp.* from infected fish: total of 100 infected *C. carpio* (weight 60±3.50 g; length 20± 2.000 cm) that showed cottony/woolly, white lesion and ulcerations over the skin were collected from aquaculture in Babylon/Iraq. These fishes were brought to the laboratory in sterilized polyethylene bags for further inspection. The isolation and identification of fungi was done in Mycology laboratory in the College of Veterinary Medicine/University of Baghdad from December 2017 until April 2018. The infected collected fishes were kept separately in aquaria with continuous aeration. The infected areas were excised and rinsed three times with sterilized distilled water. Then, these infected areas were transferred to a sterile Petri dish containing 15 ml of autoclaved distilled water. Later on, sterilized sesame seeds (5-7 seeds/petri dish) were added as a bait substrate. After that, the plates were incubated at 20˚C and were colonized by spores or mycelia for 24-72 h. Single colonized sesame seeds were aseptically transferred to autoclaved Sabouraud Dextrose agar (SDA). The medium was augmented with antibiotics [(Ab-SDA), (Chloramphenicol at concentration of 0.05 g/L)] to prevent bacterial contamination and to obtain a pure culture. Then, culture plates were incubated for 3-5 d at 20˚C, before a transfer of a mycelia plug from the edge of the colony to a fresh Ab-SDA plate and observed for hyphal growth. 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 haemocytometer according to protocol described by Horwitz *et al.* (15).

Test chemical (hydrogen peroxide): The test chemical for this study was hydrogen peroxide (H_2O_2) at concentration of 35% active ingredient. It was obtained from Sigma. All test concentrations of hydrogen peroxide were selected based on a pervious study by Rach *et al.* (26). Treatment doses were prepared by dissolving a calculated volume of hydrogen peroxide in a specific volume of well water sufficient to prepare a bath solution for treating fish for 30 min. Treatment concentrations were corrected for specific gravity and all concentrations are reported as a volume to volume ratio, e.g., ml/L.

Fish maintenance and experimental design

A total of 100 of healthy *C. carpio* (50 ± 2.0 g. B.W) were obtained from a private fish pond at Iraq/Babylon province. All fishes were acclimatized for two weeks before starting of the trial. Fish were kept in prepared glass aquaria (70 ×50 ×35 cm) filled with tap water. These aquaria were supplied with oxygen sources and de-chlorinated tap water. Continuous aeration was maintained in each

aquarium using an electric air pump. The pH, DO and water temperature were maintained at 7.5–8.0 6.5-7 mg/l and 24 ºC, respectively. ====Fish were fed on a commercial fish diet at daily rate of 1% body mass throughout the experiment. The photoperiod during holding and trying was roughly 12 h of light followed by 12 h of darkness. After acclimatization, fish were divided into five equal groups, 20 fish for each group. The first group served as control group (divided into positive control infected with Saprolegnia and negative control healthy fish). The second, third, and forth groups (T2, T3, T4); fish were infected with *Saprolegnia spp*. at concentration of $2x10^4$ zoospore/L (The fungi isolates were introduced to the fish aquarium and left in the aquarium until the appearance of the infection). Then fish treated with H_2O_2 at concentrations of 1, 3, 6 ml/L for 30 min. for 3 successive respectively; The fifth group (T5); fish were infected with *Saprolegnia spp*. at concentration of $2x10^4$ zoospore/L and treated with formalin (0.15 ml/L) for 60 min for 3 successive d interval. Formalin was used as a reference treatment to test their potency in controlling the fungal infection.

Histopathological examinations : After 15 d from the treatment with H_2O_2 and formalin, six fish for each treatment group were dissected and tissues (gills and skin) were collected for histopathological investigation. Tissues were fixed in formaldehyde solution (10%) for 48– 72 h. After fixation, the tissues were washed in water, dehydrated in graded ethanol solutions, cleared in xylene and embedded in paraffin. Paraffin blocks were sectioned 3-7 µm thickness using a microtome (Microm HM 340E). Then, the tissues then were stained with haematoxilin and eosin (HE). Slides were inspected under the light Microscope to study the variations. Gills and skin lesion scoring was carried out according to Burnet *et al.* (4) with some modifications by Mustafa (20). A quantitative evaluation of lesions in a histopathological examination was done through practical statistics (ANOVA). A probability level equal or less than 5% ($P < 0.05$) was considered significant.

Survival rate : Survival rate was calculated after seven d from the infection with Saprolegniasis and treatment with H_2O_2 according to the following equation: *Survival rate (%) =final number of fish survivor/initial number of stocked fish ×100.*

RESULTS AND DISCUSSION

Histopathological examinations Gills

Fishes of the negative control group (C-) showed normal gill structures without any significant histopathologic lesions (Fig. 1A). Fish of the *Saprolegnia spp.* infected group (C+) exhibited epithelial lifting (Fig 1B). There was multifocal and focal fusion of the secondary gill lamellae due to hyperplasia of the basal epithelium of the primary lamellae with mononuclear cells (MNCs) infiltration and also there was dilation in the central venus with blood congestion (Figure 3C). Also, positive control showed necrosis of the primary and secondary gill lamellae (Fig 1D). Similar alterations were seen in fish infected groups and treated with H_2O_2 or formalin (T2, T3, T4 and T5) but are significantly decrease compared to positive control group (Table 1). These changes including necrosis of the primary lamellae, lamellar, multifocal and focal fusion of the secondary gill lamellae due to hyperplasia of the basal epithelium of the primary lamellae and aneurysm was evident. (Fig. 2 E, F and G). Additionally, there was mononuclear infiltrations (MNCs) at the base of the primary gill lamellae (Fig. 2H). According to the observations in the current study, Saprolegnia fungi cause major histomorphological alterations in the gill of *C. carpio* such as hyperplasia, partial and completely fusion of secondary lamellae. These changes were consistent to those found by Ashour *et al.* (2) and Hamad and Mustafa (11) who observed that the common carp infected with *Saprolegnia sp.* exhibited multifocal and focal fusion of the secondary gill lamellae due to hyperplasia of the basal epithelium of the primary lamellae. These alterations are examples of general defense mechanisms (i.e. protective function). These changes might result in the increase of space between the external environment and blood which act as a barrier. On the contrary, fusion of secondary lamella decreased inter lamellar space, leading to reduction in the diffusion conductance of the gills to respiratory gases (10, 22), Result in reduced oxygen exchange by gills. Similar changes were also observed

by other researchers (7,19,25). Telangiectasis of secondary lamellae was produced as a response to break down of blood vessels integrity due to damage of pillar cells and pooling of blood (9).In fish treated with hydrogen peroxide groups there was severe mononuclear cells aggregation in the base of the primary lamellae, mainly neutrophils and lymphocytes; similar to studies carried out by Bennet and Bennet (3). The presence of high number of inflammatory cells could be attributed to host cellular reaction or as an immune response.

Skin : Fishes of the negative control group (C-) showed normal skin structures without any significant microscopic lesions (Fig. 3A). Various types of alterations were seen in skin sections of the positive control such as loss of epidermal layer with complete necrotizing of dermis and hypodermis also were seen, with cellular debris and fungal hyphae and severe vacuolation filled with fungal material (Fig. 3B). Similar changes were observed in skin of treated groups (T2, T3, T4 and T5) but the intensity of lesions were significantly decrease compared to positive control group (C+). Also, the skin of treated groups with hydrogen peroxide or formalin showed an increase of MNCs (monocytes and lymphocytes) infiltration and slight sloughing of the most superficial layer of the epidermis, which indicating the disappearance of the causative agent (Fig.3 C- F& Table 1). Histopathological alterations in skin and muscles of *C. carpio* are in consistent with results of Ashour *et al.* (2). Hamad and Mustafa (11) who observed complete erosion and ulcerative of epidermis associated with MNCs aggregation. These changes could be represented the increase of enzymatic action as a result of *Saprolegnia sp.* infection. Fregeneda-Grandes (8) have established that these alterations in skin due to proteolytic enzymes secreting by *Saprolegnia spp*. Peduzzi and Bizzozero (21) have also demonstrated that the thalli of pathogenic strains of *Saprolegnia* showed chymotrypsin-like activity and proved that this enzymatic activity could be a causative factor to the pathogenesis of Saprolegniasis. Similar observation were also seen by other workers (1, 6). Similar pattern of changes in the skin of Saprolegnia infected fish have been also described by Chauhan *et al.* (5), Hatai Hoshiai, (12) and Hussian *et al.* (17).

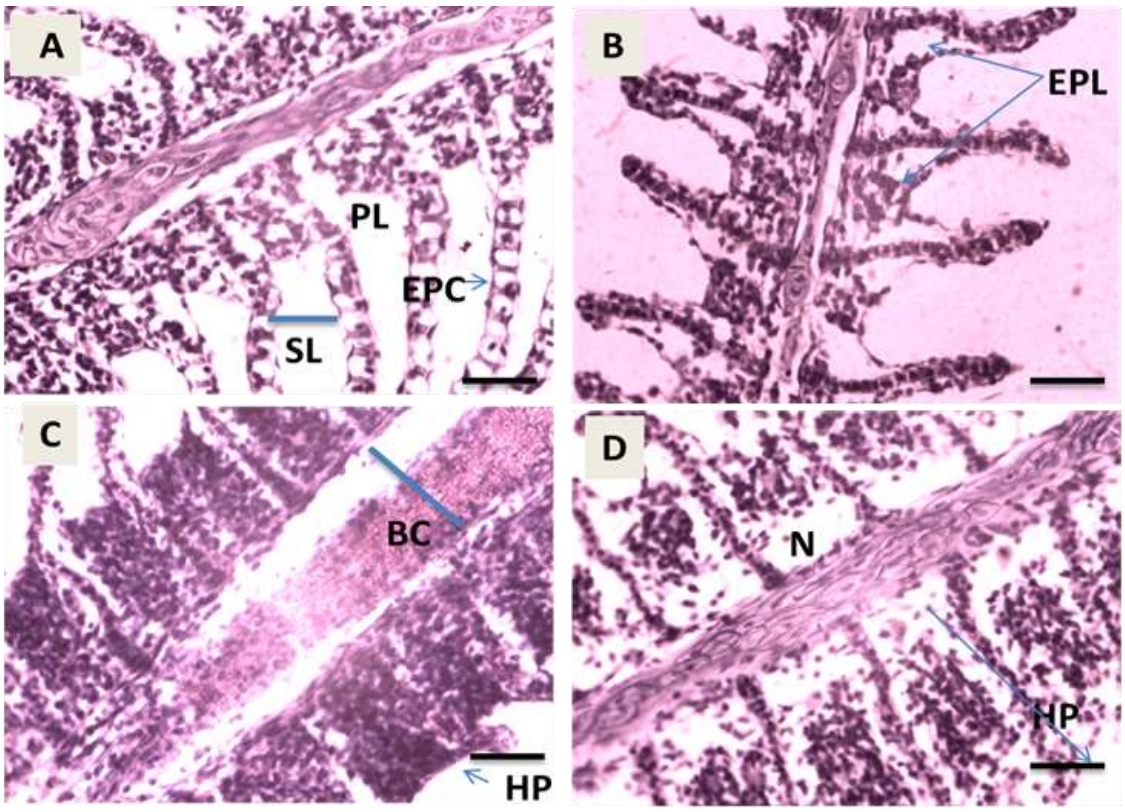

Figure1. Photomicrographs of the gill of *C. carpio* (A) control gill showing the primary lamella (PL), secondary lamella (SL), epithelial cells (EPC); (B) positive control showing epithelial lifting (EPL); (C) hyperplasia of the basal epithelium of the primary lamellae (HP) dilation in the central venus with blood congestion (BC); (D) necrosis with mononuclear cells (MNCs) infiltration. H&E; x400, Scale bars 50µm. Thickness 3-7 µm.

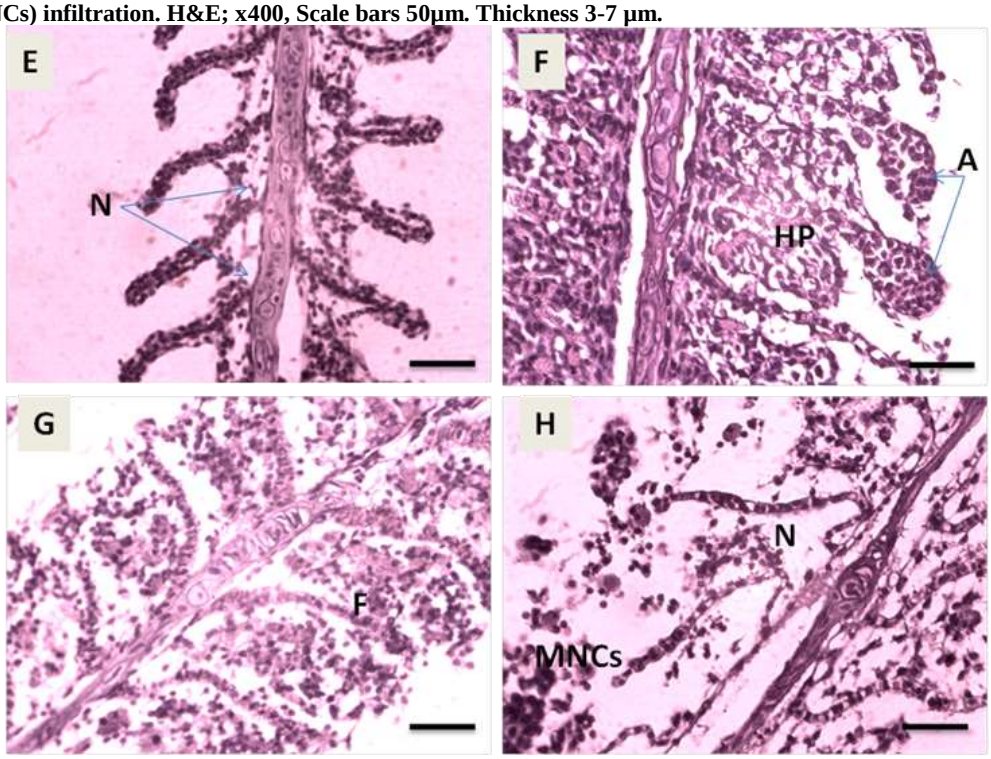

Figure 2. Photomicrographs of the gill of *C. carpio* (E-H) gills of infected groups and treated with hydrogen peroxide or formalin (T2, T3, T4 and T5) showing necrosis (N), hyperplasia (HP), aneurysm (A), fusion of the secondary gill lamellae (F), necrosis (N) with mononuclear cells (MNCs) infiltration. H&E; x400, Scale bars= 50µm. Thickness 3-7 µm.

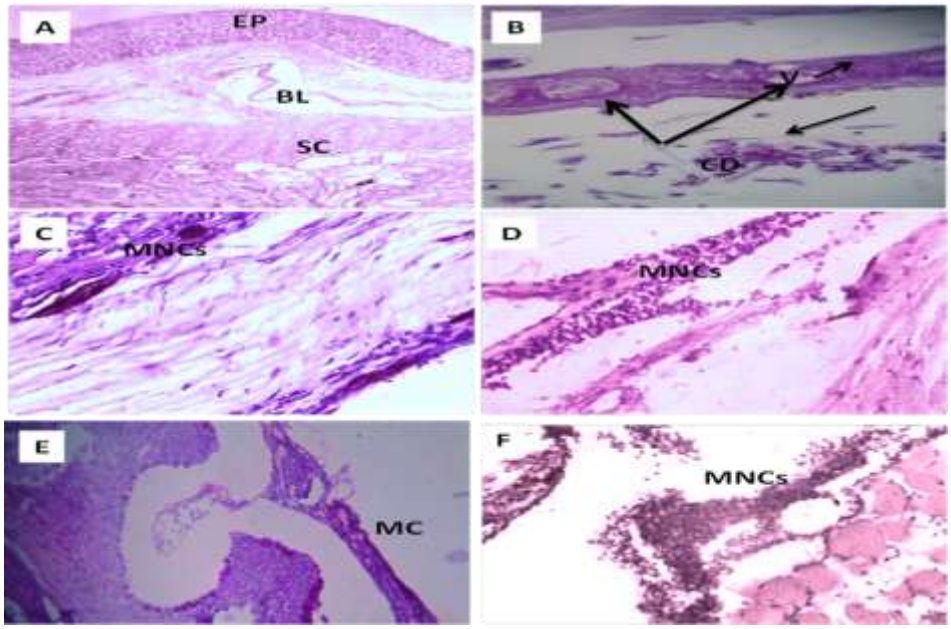

Figure 3. Photomicrographs showing histological structures through skin of *C. carpio* infected with *Saprolegnia sp.* and treated with hydrogen peroxide and formalin (A): control showing epidermal layer (EP), basal layer (BL) and stratum compactum (SC) 10x; (B): positive control showing complete loss of epidermal layer with cellular debris (CD) with fungal hyphae with severe vacuolation filled with fungal material (V); (C-F): Treated groups showing mononuclear cells infiltration (MNCs) with increased number of mucous cells (MC). H&E; Scale bars: A=25 µm (x100); B-F= 50µm (x400). Thickness 3-7 µm.

Table 1. Histopathological alterations as a percentages in gills and skin of the *C. carpio* infected with *Saprolegnia spp*. and treated with H_2O_2 or formalin (Burnet *et al.* 1999).

Organ/Tissue	C-	C+	T2	T3	T4	T5
Gills alterations						
Fusion of the secondary lamellae	0.00±00.0a	50.03±4.09b	32.5±3.00c	29.00±5.00d	31.00±2.00c	37.00±4.00c
Epithelial proliferation	2.30±0.50a	42.07±6.04b	31.50±2.00c	33.00±4.00c	35.00±3.00c	30.00±2.00c
Epithelial lifting	0.00±0.00a	52.08±5.30b	25.60±4.00c	21.00±3.00c	18.00±2.00c	22.00±4.00c
Necrosis	0.00±0.00a	37.09±4.50b	18.00±4.00c	25.00±3.00c	21.00±2.00c	30.00±2.00d
Aneurysm	0.00±0.00a	12.80±2.40b	20.00±2.00c	3.00±0.60d	6.00±1.00d	02.00±0.50d
MNCs infiltration	2.00±0.06a	19.32±3.50b	45.00±6.00c	47.00±5.00c	36.00±7.00d	34.00±5.00e
Skin alterations						
MNCs infiltration	0.00±0.00a	12.93±4.00b	33.00±4.00c	32.5±3.00c	34.6±5.00c	39.00±6.00d
Damage of epidermal layer with cellular debris	0.00±0.00a	43.06±5.60b	27.00±5.00c	33.00±5.00c	25.00±3.00c	30.00±4.00c
Vacuolation	0.00±0.00a	36.07±4.60b	0.00±0.00 b	0.00±0.00 b	0.00±0.00 b	0.00±0.00 b

Values are mean±SE. Different alphabetic letters horizontally are indicated significant differences at P<0.05

Table 2. Percentage of survival for all treatment groups of *C. carpio* infected with *Saprolegnia spp.* and treated with different concentrations of H_2O_2 and formalin.

Groups		Control groups		H_2O_2 treatment groups			Formalin treatment group
		C-	C+	T2	T3	T4	T5
				6	3		
No. of fish		10	10	20	20	20	20
Follow up 15 days	Dead	0	8	2	5	7	4
	Survive	10	2	18	15	13	16
Survival (%)		100%	20%	90%	75%	65%	80%

Survival rate : Following treatment with H_2O_2 (i.e., 15 d) percentage of survival of *C. carpio* was calculated. Fishes that died were found to be infected with *Saprolegnia spp.* at the time of examination. Fish of the C+ group experienced 20.0% survival rate by test end. The second group (T2) at concentration of 1ml/L H_2O_2 offered the highest survival rate (90%) followed by T3 and T4 (75 and 65%) respectively. Formalin group (T5) showed survival rate reached up to 80%. No infection or mortality was observed in any fish of the control negative group. (Table 2). Both H_2O_2 treatments of 3 and 6 ml/L obviously caused some treatment- related mortality. Higher concentration of hydrogen peroxide (6 ml/L) treatment produced higher mortality related to concentration of 1 and 3 ml/L. This concentration (6ml/L) could be to additional chemical stress to fish, that when combined with stressors of the Saprolegnia infection model, leading to higher mortality with exposure concentration increase. As a result, this study showed that H_2O_2 the most effective method for controlling Saprolegniasis in common carp at concentration of 1ml/L as observed through the survival rate and results of histopathological studies Howe *et al.* (16) reported that 7.5ml/L of H_2O_2 effectively controlling mortality, reducing the incidence of infections, and enhancing the recovery of infected channel catfish. Although the use of H_2O_2 did not completely remove fungal infection, it could decrease the frequency of the use of malachite green which is known for its threat to the environment. However, to introduce H_2O_2 as a fungistatic compound in fish aquaculture. the current effort will certainly help to understand the severity of fungal infection in the production system and thus to take successful plans to increase the production of common carp in the aquaculture. The use of H_2O_2 in aquaculture is growing and there is a require to develop essential guidelines to effectively treat diseased fish. The result of the present study also indicated that the histology could be an effective tool in describing the alterations in the selective tissue of common carp having fungal infection.

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