Immunological and pathological status of oreochromus niloticus and monosex-tilapia due to aeromonas Hydrophila infection

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ABSTRACT

This study was done to compare the pathological changes and immunological response between Oreochromus niloticus and monosex tilapia after Aeromonas hydrophila injection. Both fish species were experimentally infected with localy isolated *A. hydrophila*. The lethal dose fifty (LD_{50}), clinical sings, post mortem lesions, histopatholological changes, phagocytic activity and antibody titers against injected bacterin were examined. The obtained results of LD_{50} in *O. niloticus* and monosex tilapia were $10^{3.5}$ and $10^{3.7}$ respectively. Same clinical signs and PM lesions in both species in the form of dark skin, exophthalmia, abdominal distension (ascitis) , fin erosion and hemorrhagic patches at base of fins and fish body were observed. Upon necropsy, the gill appeared congested, enlarged, enlarged paleness of the liver, with multifocal white area, distended gall bladder, and congestion of spleen and kidneys were found. Significant reduction of lymphocytes and monocytes numbers were recorded in both fish species after 2 weeks postinjection. Phagocytic activity was decreased in both species, but the PA in *O. niloticus* was lower than PA in monosex tilapia. Both species responded positively to injected bacterin and gave high antibody titer. Regarding to the histopathological lesions ,the activation of MMCs and lymphocytic infiltration were the most important signs detected in spleen and kidneys in both species .

INTRODUCTION

Fish play an important source of animal protein worldwide and increasing role in solving the shortage of animal protein especially in Egypt. Oreochromus niloticus is one of the most widely cultured fish in the world, with more attention in Egypt (Young and Muir 2000 and GAFR 2002).

In tilapia culture, the production of all male population through treatment of fry with 17 a-methylestostrone (MT) – impregnated food had become the most popular procedure in Egyptian aquaculture. All male population has greater potential because no energy is shunted toward reproduction and no competition with younger fish occurs (Green et al., 1997).

The potential advantages sought from the production of all male population may include one or more of the following features: achievement of the higher average growth rate, elimination of reproduction, reduction of sexual/territorial behaviors, prevention of variation in harvest size and reduction of environmental impact resulting from escapes of exotic species (Beardmore et al., 2001).

Fish production is greatly affected by bacterial infection specially Aeromonas hydrophila. It's the causative agent of Motile Aeromonas Septicemia (MAS), one of the most serious and frequently encountered pathogens of fish in warmwater aquaculture (Soliman, 1988) specially in Egypt.

This study was planned to compare the pathological changes and immunological responses between O. niloticus and **monosex tilapia after** experimental infection with local isolated A. hydrophila.

MATERIALS AND METHODS

Determination of lethal dose fifty (LD_{50})

A total number of 84 apparently healthy fish average weight 70 ± 5g. (42 *O. niloticus* and 42 monosex tilapia) which previously proved to be free from *A. hydrophila* by re-isolation from some fish stock and proved to be negative according to the criteria of (Davis *et al.* 1980) were used.

Fish were divided in to 7 groups (6 fish/group) from each fish species and kept in glass aquaria supplied with chlorine free tap water and oxygen and fed 25% protein diet. The first 6 groups from each species were injected I/P with serial ten fold dilution of locally isolated *A. hydrophila* (Kindly provided by Dept of Poultry and Fish Disease, Fac. of Vet. Med. Alex. Univ.), while, the seventh group were injected with sterile saline and served as control.

The LD_{50} was determined according to (Reed and Munch, 1938). The fish were kept under observation for 7 days.Re-isolation of the inoculated bacteria from freshly dead fish was done for verification of death in this experiment and other one.

Experimental infection :

A total number of 72 apparently healthy fish average weight 70 ± 5 g. (36 *O. niloticus* and 36 monosex tilapia) were divided randomly into 9 groups (8 fish/group). Fish in the first 6 groups were injected with 0.2 ml containing of (5×10^3 cfu/ml) according to Shehata *et al.,* (1985). The last 3 groups were injected with 0.2 ml of sterile saline and served as control.

The clinical signs and mortality were recorded during the experimental period (8 weeks). Necropsy was done from 3 randomly fish from each group and samples from liver, kindneys, gills, spleen and intestine were collected from each fish after 2, 4 and 8 weeks. Blood samples were collected at the same time and used for the following studies:

1. Differential leucocytic count

The blood were collected from caudal artery for determination of differential leucocytic count according to the method of (Lucky 1977). The

absolute value and percentage of each type of cells were calculated according to (Schalm 1986).

2. Phagocytic activity and phagolytic index

Phagocytic activity was done according to (Kawahara et al., 1991 and Soliman 1997). Fifty mg Candida albicans was added to/one ml of citrated blood collected from 3 fish from injected and control groups of each species and shaken in water bath at 25 C for 5 hrs. Blood smears were stained with Giemsa. Phagocytosis was estimated by determine the proportion of macrophages which contained intracellular yeast cells in a random count of 300 macrophages and expressed as percentage of phagocytic activity (PA). The number of phagocytized organisms were counted in each phagocytic and called phagocytic index (PI).

Immunological respose:

A. hydrophila was used in the preparation of formalin killed bacterin according to (Sakia et al., 1984).

The bacterin was mixed with an equal volume of 0.85% sterile salin. The bacterial number was adjusted to Macfarland's No. 2 (6 x 103 Cells/ml). (Badran, 1990). Twenty fish from each species were injected IP with 0.2 ml bacerin/fish. Equal number from each type were injected with 0.2 ml/fish with sterile saline and served as control. After 2 weeks, the treated fish were injected with a booster dose of bacterin (the same dose). Serum samples were collected after 4 weeks and stored at -20C until use (Lucky, 1977). The immune response to A. hydrophila bacterin were evaluated by micro test according to (Badran 1990. Agglutination titers were expressed as every week log2 of the highest serum dilution still giving a clear agglutination.

All the results of previous work were expressed as means ± (S.E) standard error and differences were evaluated by Student's F-test.

2.4. Histopathological studies

The collected tissue samples from infected fish were fixed in 10% neutral buffered formalin solution. The fixed samples were processed and stained by Hamatoxyline and Eosin (H & E) according to (Culling 1983).

RESULTS

1- Results of experiment

The results of A. hydrophila in O. niloticus and monosex tilapia were 103.5 and 103.7 respectively.

2- Results of experiment

Clinical signs and postmortem lesions in fish injected with A. hydrophila were almost the same in both species of fish.

Fish sink down to the bottom of the aquaria and grouped together. The skin of affected fish appeared dark (Fig. 1). In addition, unilateral exophthalmia (Fig. 2) and abdominal distention (Fig. 3) were noticed in some fish . Hemorrhagic patches at base of the caudal peduncle with congestion of rays of the caudal fin were noticed (Fig. 4). Furthermore, there were fin erosions with development of different sized and shape ulcers on both sides of body surface. Upon necropsy, the gills appeared congested with marked swelling. Liver was enlarged and pale contained multifocal white area with distended gall bladder with greenish bile. Moreover, the spleen was congested and enlarged with presence of small amount of sanguineous fluid in the abdominal cavity (Fig. 5). Furthermore, the intestine of some fish was filled with yellowish mucous (Fig. 6). The post kidney was soft in texture.

Differential leucocytic count:

After 2 weeks post-injection of monosex tilapia with A. hydrophila significant reduction of lymphocytes, monocytes and eosinophiles were noticed. While after 4 weeks of injection, there were significant reduction of some types of cells plus basophiles . At the end of experment there was significant reduction in basophiles Table (1).

While in case of O. niloticus there were significant reduction of lymphocytes, monocytes and eosinophils and significant increase of neutrophils after 2 weeks of injection. While after 4 weeks of injection there were significant reduction of lymphocytes, monocytes and basophiles, and significant increase of neutrophuls. Significant reduction of monocytes and significant increase of basophiles were recorded at the end of the experiment Table (2).

Table (1): Differential leucocytic counts in monosex tilapia injected with *A. hydrophila*.

Periods	Group	Lymphocytes	Monocytes	Basophiles	Eosinophils	Neutrophils
0 day		53.33±0.33C	1.33±0.33C	7.67±0.88E	12.00±0.58A	25.33±1.76D
2 Week	Injected	47.33±2.03E	0.67±0.33D	6.67±0.33F	12.00±0.85A	26.67±1.20CD
	Control	51.33±0.88D	1.67±0.33C	9.33±1.20C	13.00±0.58A	23.00±0.58D
4th week	Injected	42.67±2.33E	1.33±0.33C	8.67±0.33D	7.76±0.33F	28.00±2.00C
	Control	53.67±1.76C	2.33±0.33B	11.00±0.58A	10.33±0.40C	26.00±0.58CD
8th week	Injected	57.00±1.73 B	2.00±0.58B	8.00±0.58D	10.67±0.33C	22.67±0.03D
	Control	58.67±3.18B	2.00±0.58B	9.67±0.88C	10.33±0.88C	23.33±2.60D

Means within the same column carrying different letters are significantly different at ($P < 0.01$).

Table (2): Differential leucocytic counts in *O. niloticus* infected with *A. hydrophila*.

Periods	Group	Lymphocytes	Monocytes	Basophiles	Eosinophils	Neutrophils
0 day		53.33±0.33C	1.33±0.33C	8.33±0.88D	11.33±0.33B	22.67±1.45D
2 Week	Injected	47.33±2.03E	0.67±0.33D	6.67±0.33F	12.00±0.85A	29.67±0.33C
	Control	51.33±0.88D	1.67±0.33C	9.33±1.20C	13.00±0.58A	23.00±0.58D
4th week	Injected	42.67±2.33E	1.33±0.33C	8.67±0.33D	7.76±0.33F	26.00±0.58CD
	Control	53.67±1.76C	2.33±0.33B	11.00±0.58A	10.33±0.40C	18.33±2.60E
8th week	Injected	58.00±0.08B	1.67±0.33C	8.67±0.33D	12.00±0.58A	19.00±0.58E
	Control	59.00±1.15B	2.67±0.33B	7.67±0.33E	12.33±0.33A	18.00±0.58E

Means within the same column carrying different letters are significantly different at ($P < 0.01$).

The phagoytic activity and phagocytic index:

The results of PA and PI are summarized in Table (3) and Fig. (7).

From the results there was significant decrease in phagocytic activity after 2 weeks of injection, Meanwhile, phagocytic index showed a significant increase after 4 weeks and 8 weeks of injection as compared with control group.

There was significant decrease of phagocytic activity after 2 and 4 weeks of injection in contrast to phagocytic index which increased after 2 and 4 weeks.

Table (3): Phagocytic activity and phagocytic index in monosex tilapia and *O. niloticus* infected with *A. hydrophila*.

Periods	Group	PA	PI
0 day		22.33±0.88D	3.53±0.27C
2 Week	Injected MT *	18.33±1.45G	3.63±0.43C
	Control MT	22.33±0.88D	3.54±0.38C
	Inject ON **	18.00±0.58G	4.34±0.13B
	Control ON	23.67±1.20C	2.63±0.53D
4th week	Injected MT	21.67±1.45D	4.50±0.40B
	Control MT	22.00±1.73D	3.04±0.16C
	Inject ON	23.33±0.88C	3.30±0.20C
	Control ON	24.33±0.88B	2.46±0.34D
8th week	Injected MT	24.33±0.33B	4.00±0.42B
	Control MT	24.33±1.45B	3.83±0.33C
	Inject ON	23.33±0.88C	3.37±0.00C
	Control ON	24.33±0.88B	3.82±0.02C

Means within the same column carrying different letters are significantly different at ($P < 0.01$).

*MT= Monosex tilapia.

**ON= *O. niloticus*.

Antibody Titer

Antibody titer after 4 weeks in case of monosex tilapia and *O. niloticus* were almost the same ,namelly 6.00 ± 0.58 and 6.00 ± 0.33 $\log_2$ in case of O.niloticus and monosex –tilapia respectively .

Histopathological changes due to infection with *A. hydrophila*

Second week of the experiment:

Liver:

Diffuse moderate hydropic degeneration with melanophores deposition (Fig. 8) were noticed in *O. niloticus* with or without congestion of portal blood vessels. The noticed lesions in monosex tilapia resembled that previously described, in addition to lymphocytic infiltration besides congested hepatic sinusoids.

Gills:

The gills of both O. niloticus and monosex tilapia were similar in nature. These lesions were diffuse clubbing at the tips of the gill filaments with edema of the gill arch (Fig. 9).

Posterior Kidney:

The post Kidney of O. niloticus exhibited focal necriotic area severely infiltrated with lymphocytes (Fig. 10). In monosex tilapia lesions were similar to that previously described in addition to activation of MMCs with EGCs infiltration .

Spleen:

The spleen of O. niloticus showed lymphocytic cell necrosis with hyperactivation of MMCs. In monosex tilapia, there was activation of MMCs surrounded with thick walled fibrous tissue capsules.

Intestine:

The intestine of O. niloticus showed necrotic enterities with desquamation of the necrotic mucosa within the lumen.

Fourth week of the experiment:

Liver:

The only noticed lesion in O. niloticus was mild hydropic degeneration. In addition to the aforementioned lesion, there was intracellular deposition of the melanin pigments inside the hepatoytes especially around bile duct of monosex tilapia (Fig. 11).

Gills:

Generally, the noticeable lesions in the gills of both O. niloticus and monosex tilapia were nearly similar to that previously described in the cases of 2rd week, in addition to congestion of the bronchial vessels.

Edema with congestion besides lymphocytic infiltration in the gill arch was noticed.

Kidneys:

The lesion of both O. niloticus and monosex tilapia were hydropic degeneration of the tubular epithelium with tubular and glomerular necrosis (Fig. 12).

Spleen:

The most splenic alteration of O. niloticus was multifocal necrosis of white pulps which replaced by fibrous tissue represented by honeycombed appearance. Regarding to monosex tilapia, the lesions were represented by multifocal lymphocytic depletion represented by presence of minute spaces with hyperactivation of MMCs.

Eighth week of the experiment:

Liver:

The noticeable lesion in the liver of both O. niloticus and monosex tilapia, were nearly similar in nature. These lesions were congestion of the major portal vessels with hemorrhage of the hepatic parenchyma.

Gills:

In both of O. niloticus and monosex tilapia, the gills exhibited edema and EGCs infiltration within the gill arch.

Kidney:

The only detectable lesion in the Kidneys of both of O. niloticus and monosex tilapia, were hyperactivation of MMCs.

Spleen:

Activation of MMCs was the only recorded lesion in both O. niloticus and monosex tilapia.

DISCUSSION

Bacterial diseases are responsible for mortality in both wild and cultured fish (Robert, 1989). Aeromonas hydrophila is opportunistic pathogen for fish and cause high mortality under culture conditions (Faisal et al. 1989 and Cipriano 2001). In this study both immunological and pathological parameters in O. niloticus and monosex tilapia due to experimental in fection with A. hydrophila were monitored. The lethal dose 50 (LD50) in O. niloticus and monosex tilapia were 103.5 and 103.7 respectively. These results are agree to those reported by (Faisal et al. 1989). The difference between the LD50 of both species may be attributed to the more resistance of monosex tilapia to A. hydrophila than O. niloticus. The same conclusion was reported by (El-Gamal 2005). In case of A.

hydrophila infection, the clinical signs and postmortem alterations were similar in both monosex tilapia and O. niloticus. Clinically, the affected fish were lethargy and suffered from darkness of the skin, hemorrhagic patches on the body surface especially at the caudal peduncle with congestion of the caudal fin. Unilateral exophthalmia and abdominal swelling. In addition, different sized and shaped ulcers on both sides of body surface were noticed. These lesions may by due to liberated exotoxin (cytotoxin and protease) of the injected bacteria. Similar results were obtained by Cone, 1982; Mohamed 1990, Roberts, 2001. The postmortem examination of the cases of A. hydrophila infection revealed congested gills with marked swelling. The liver was enlarged and pale contain multifocal grayish white areas. Furthermore, marked splenomegally with congestion were noticed. The kidney appeared soft and the intestine was filled with yellowish mucous. The gall bladder was several times its normal sized and distended with greenish bile. Presence of small amount of sanguineous fluid was noticed in the peritoneal cavity. These changes may by due to the liberated exotoxin of the injected bacteria which destructed the tissue cells (Easa et al. 1985; Mohamed 1990; El-Abbasy 1994; Doukas et al. 1998 and Afifi et al. 2000). The microscopical examination of the liver of these cases revealed hydropic and/or fatty change of the hepatocytes. Moreover, there was multifocal hepatic and pancreatic necrosis with activation of MMCs. Congestion of the hepatic blood vessels and sinusoids was noticed. These observation may be due to bacterial cytotoxin. Sometimes, lymphocytic and EGCs infiltration was seen, these cellular reaction may be considered as line of defense mechanism (Doukas et al. 1998; Noga 1996; Aoki 1999 and Afifi et al. 2000). In some cases, there were diffuse lyses of the hepatocytes and complete loss of structural integrity, which may due to cytotoxin liberated by the inoculated bacterium. These observations are completely agree with (Huizinga et al. 1978). The gills of these infected fish showed diffuse filamentous clubbing and hyperplasia of the epithelial lining at the base of secondary gill lamellae, These proliferative changes might by considered as a reaction against infection. There were focal necrosis, lymphocytic infiltration, telangiectasis and edema at the base of secondary lamellae with or without separation between surface epithelium and capillary bed of some cases which may be due to increase capillary permeability. Moreover, congestion and EGCs besides lymphocytic infiltration within the gill arch. These observations are partially correlate with those reported by (Soliman 1988 and Ventura and Grizzle 1988). The kidneys exhibited proteineous dystrophy in the form of cloudy swelling and hydropic degeneration of the tubular epithelium. Furthermore, glomerular and tubular necrosis with lymphocytic infiltration was noticed. These

observations may by attributed to the liberated cytotoxin of the injected bacterium. Hyaline droplet in the tubular epithelium and eosinophilic detritus in their lumina were noticed especially in monosex tilapia. The results may by due to affection of the glumeruli which become more permeable to plasma protein even albumin. Activation of MMCs that play a role in the defense mechanism. (Soliman 1988; Miyazaki et al. 2001 and Roberts 2001). The splenic alterations were multifocal lymphoid necrosis and depletion besides hyperactivation of MMCs. Moreover, hemopoietic tissues and red pulp were diminished. This observation may be due to the liberated exotoxin of the inoculated bacteria. These results are coordinate with Huizinga et al. (1978); Bowser 1999 and Roberts (2001). The intestinal submucosa showed congestion, edema besides lymphocytic infiltration, which may play a role in defense mechanism. Furthermore, necrosis and denudation of intestinal mucosa which may result from action of the liberated cytotoxin of the inoculated bacteria.

The blood parameters especially the differential leucocytic count has diagnostic importance and usually readily respond to identical factors such as physical, chemical and biological stressors (Hickey, 1976 and Soliman 1997

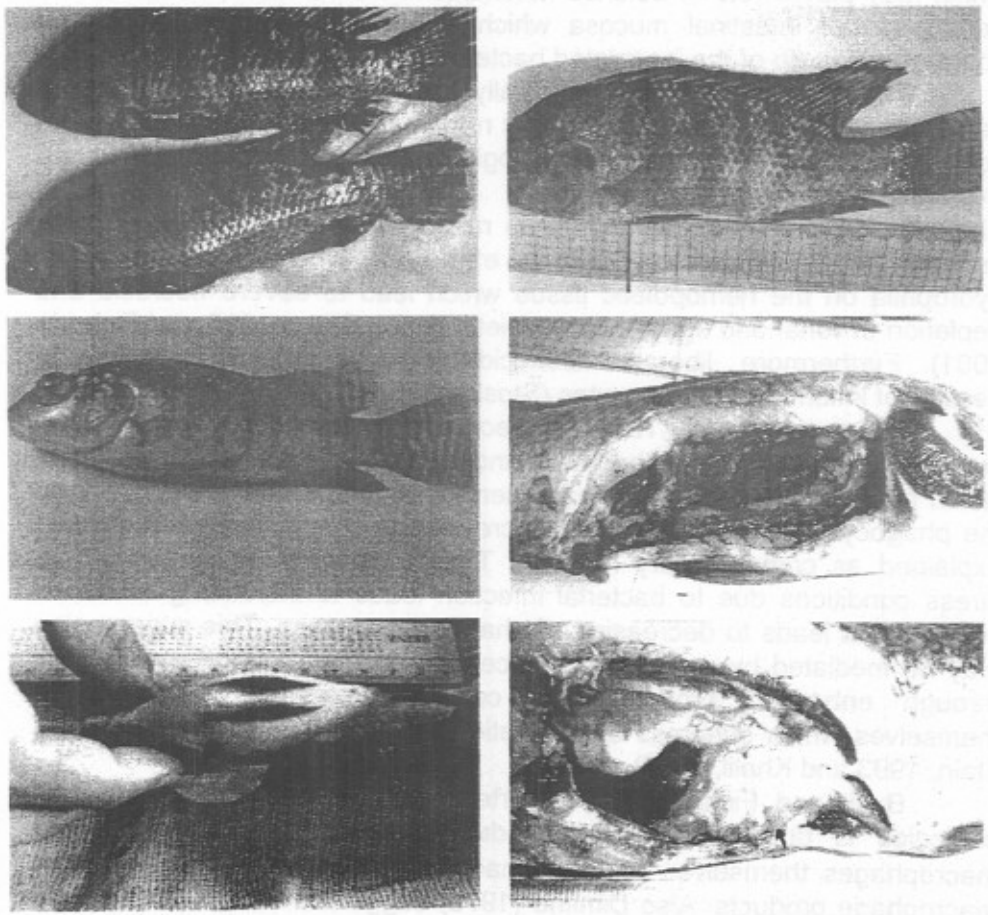
The infected cases with A. hydrophila revealed severe leucopoenia. This previous result may be attributed to the effect of the liberated cytotoxin of A. hydrophila on the hemopoietic tissue which lead to severe necrosis and depletion of renal and splenic hemopoietic tissue (Noga 1996 and Roberts 2001). Furthermore, liberated leukocidin of A. hydrophila resulting in destroy of inflammatory leucocytes (Stoskopf, 1993).

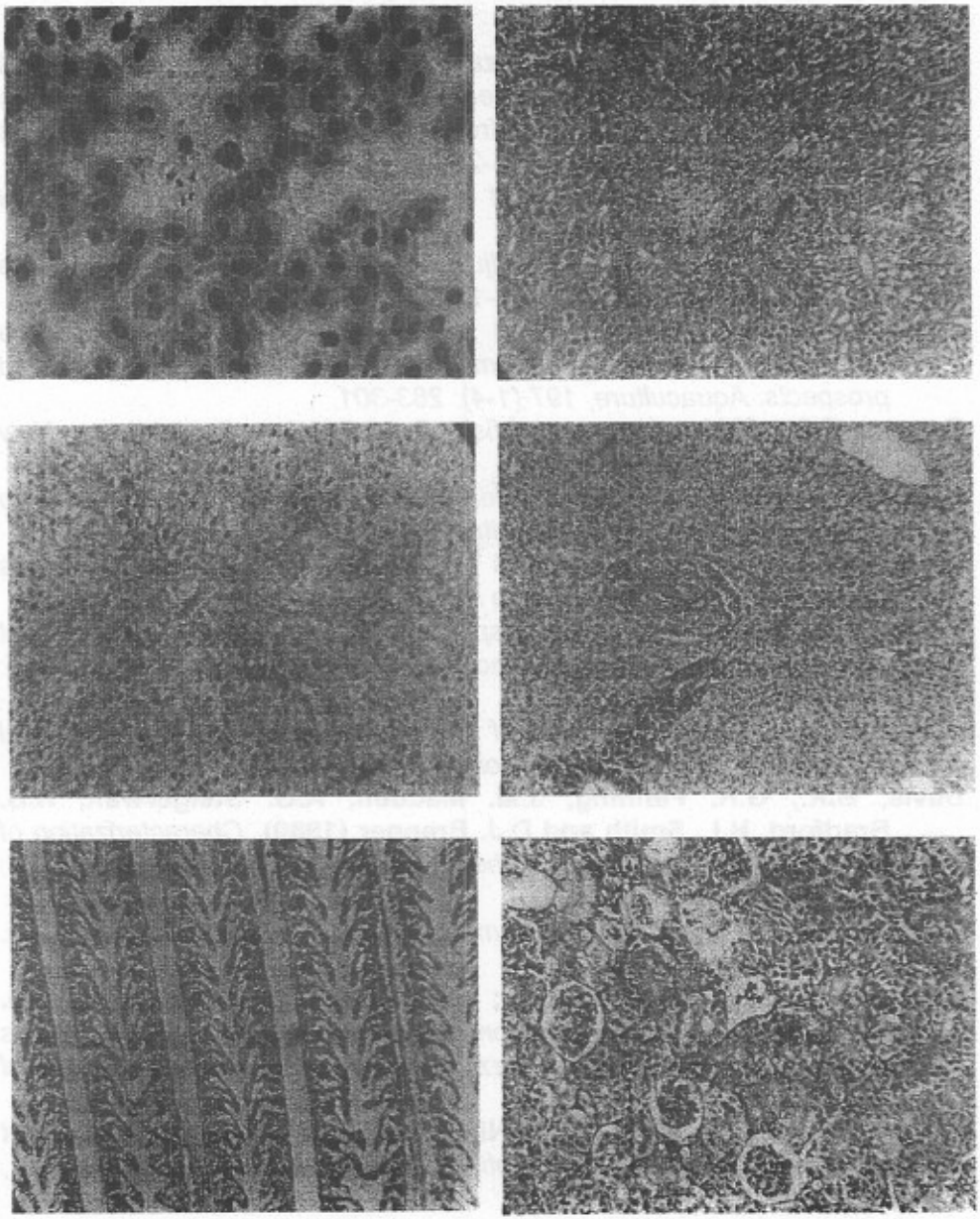
Phagocytic assay revealed decrease in phagocytic activity and some times increase the phagocytic index in both types of infected fish, which may be attributed to increase serum cortisol, which affect badly on the phagocytic activity. Meanwhile, increase the phagocytic index may be explained as compensatory reaction. These results may suggests that, stress conditions due to bacterial infection leads to increasing of serum cortisol, that leads to decreasing of phagocytic process. This suppression may be mediated by corticosteroid receptors on macrophage or indirectly through enhanced production of certain factors by macrophages themselves which suppress the secretion of other macrophage products (Jain, 1993 and Khalil, 1998).

Buck and Finlay (1979) reported that the suppression effect of corticoids is due to enhanced production of certain factors by the macrophages themselves (e.g α-2 macroglobulin) which suppress other macrophage products. Also Dimitriu (1976) suggested that, the release of Macrophage Arming Factor (MAF) by antigen stimulated T-lymphocytes

was unaffected by corticoids but their uptake by macrophage and their subsequent increase of cytotoxic activity was inhibited as increasing of serum cortisol level upon stress.

The results of antibody titer were similar in both types of fish which indicated that both types of fish proved to have equal response to bacterin while monosex tilapia gave higher phagocytic activity than O. niloticus. The possible explanation for this differences that, hormonal treatment to make sex reversal may play a role in increasing the phagocytic process. Also the results of LD50 monosex tilapia was more resistant to A. hydrophila infection than O. niloticus. A decisive answer awaits further experimention. Moreover, both types of fish expresed more or less similar clinical, PM lesions, histopatholgical changes and humoral response.





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Legend of the figures

Fig. (1): A monosex tilapia infected with *A . hydrophila* showing darkness of the skin and fins erosion.

Fig. (2): A monosex tilapia infected with A. hydrophila in showing unilateral exophthalmia.

Fig. (3): A monosex tilapia during A. hydrophila infection showing abdominal dropsy.

Fig. (4): A monosex tilapia during A. hydrophila infection showing hemorrhagic patches at the caudal peduncle with congestion of the caudal fin.

Fig. (5): A monosex tilapia during A. hydrophila infection showing distended gall bladder with greenish bile, paleness of the liver with presence of white areas on its surface. Furthermore, the spleen enlarged with presence of small amount of sanguineous fluid in the peritoneal cavity.

Fig. (6): A monosex tilapia during A. hydrophila infection showing the same lesions of the previous picture (Fig. 5). In addition to presence of yellowish mucoid intestinal content (arrow).

Fig. (7): Low level of phagocytosis in monosex tilapia after 2 weeks post injection with A. hydrophila.

Fig. (8): Liver of an O. niloticus during 2nd week of A. hydrophila infection showing diffuse moderate hydropic degeneration with melanophres deposition. H&E (X 250).

Fig. (9): Gills of a monosex tilapia during 2nd week of chronic A. hydrophila infection showing edema and distortion of cartilage of primary filaments with separation of the surface epithelium from the capillary bed (arrows). H&E (X 160).

Fig. (10): Posterior kidney of an O. niloticus during 2nd week of A. hydrophila infection showing focal necrotic area infiltrated with lymphocytes H&E (X 250).

Fig. (11): Liver of a monosex tilapia during 4th week of A. hydrophila infection showing mild hydropic degeneration of the hepatocytes with intracellular deposition of the melanin pigment within the hepatocytes around a bile duct H&E (X 160).

Fig. (12): Posterior kidney of a monosex tilapia during 4the week of A. hydrophila infection showing glomerular and tubular necrosis with hydropic degeneration of the tubular epithelium of few renal tubules H&E (X 250).

الملخص العربي

الحاله المناعيه والباثولوجيه لاسماك البلطي النيلي والبلطي وحيد الجنس المصابه بميكروب الايروموناس هيدروفيلا .

صفيناز جمعه محمد

مدرس بالمعهد القومي لعلوم البحار والمصايد – معمل الباثولوجيا المائية

أجريت هذه الدراسه للمقارنه بين التغيرات الباثولوجيه والاستجابه المناعية لاسماك البلطى النيلى ووحيد الجنس بعد عدوتها بميكروب الايروموناس هيدروفيلا معمليا .

تم دراسة الجرعه نصف المميته والاعراض الاكلينيكيه والصفة التشريحية والتغيرات الباثولوجية والنشاط الالتهامي للخلايا الاكولة وقياس معدل وجود الاجسام المناعيه المضادة لهذه العدوى.

وجد أن الجرعة نصف المميتة للبلطى النيلى والبلطى وحيد الجنس كانت $10^{3.5}$؛ $10^{3.7}$على التوالى .

وأن العلامات المرضية الخارجية كانت متشابهة فى النوعية من تحول الجلد الى اللون الغامق ،جحوظ العين ،والأستسقاء ،تآكل الزعانف مع وجود بقع نزفيه على الجسم وتحت الزعانف واحتقان فى الخياشيم .

كما لوحظ تضخم فى الكبد ولونه باهت مع بعض النقاط التنقرزية وتمدد فى الحوصلة المرارية مع احتقان الطحال والكلى .

بعد العدوى التجريبية باسبوعين كان هناك نقص فى عدد الخلايا الليمفاوية والمونوسيت.

النشاط الألتهامى فى النوعين كان أقل فى البلطى النيلى عن وحيد الجنس .

كلا النوعين أستجاب لحقن البكتريا مع أعطاء معدلات عالية للأجسام المضادة .

وقد تم دراسة التغيرات الهيستوباثولوجية المصاحبة للحقن التجريبى لميكروب الايروموناس هيدروفيلا .