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Assessment of Using Carp Pituitary Extract on Clinico-Histopathological and Immunological Status of Common Carp (*Cyprinus carpio)* Broodstocks

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Abstract: The present study was conducted to investigate the effect of Carp pituitary extract (CPE) on clincopathological, immunological parameters and histopathological alterations of *Cyprinus carpio* broodstocks. The investigated fish broodstocks were reared at the hatchery of central laboratory of Aquaculture Research in Abbassa, Sharkaia governorate, Egypt. Five out of ten fish were intramuscularly injected with two successive doses of CPE. The preliminary dose was 0.3 mg/kg body weight, followed by inductive dose of 2.7 mg/kg body weight, with 8 hours intervals. The other five fish were used as control. The results of clincopathological parameters including: RBCs, PCV, Hemoglobin, WBCs, Total protein, Globulin and A/G ratio revealed no significant differences but Albumin showed significant increase among treated group compared with control group while cortisol and glucose revealed significant increase. The Liver function (AST and ALT) exhibited no significant difference. The kidney function (Serum creatinine, Blood urea nitrogen) tests showed statistically significant difference between the two studied groups in blood urea nitrogen test only. The obtained results of immunological parameters including nitric oxide production and lysozyme activity did not show significant differences between the two groups. The histopathological examination of treated group showed mild pathological alterations in renal tubules, focal leucocytes inflammatory cells aggregation in between the tubules and activation in different stages of vitellogenic follicles in ovary compared with control group. These findings prove the safety of using CPE for spawning induction in common carp broodstocks.

Key words: *Cyprinus carpio* · Carp pituitary Extract · Clincopathological Parameters · Immunological Parameters

INTRODUCTION

For many years, fish farmers have been using hormone preparations for the artificial propagation of many cultured fish. In practice, acetone-dried pituitary has been the most commonly used agent to induce ovulation [1, 2]. A variety of hormones have been used to induce final oocyte maturation and spawning in a wide range of fish species including pituitary extracts [3, 4].

The delivery of maturational hormones to fish via injection has been successfully employed by aquaculturists to induce ovulation in various species of teleost [5]. However, improper handling and a variety of other environmental factors; may inhibit the ovarian response and induce stress in fish [6]. Thus, an effective method of inducing ovulation without the need of physical manipulation would be extremely valuable to the industry. Induced spawning in common carp is released by injecting carp pituitary extract (CPE) during the spawning period. Using CPE may be simply processed or calibrated containing a predetermined dose of gonadotropin (GnRH) [7].

It is now a successful practice in cyprinid aquaculture to induce ovulation using the classical carp pituitary extract (CPE) procedure which typically involves two injections of 0.3 and 2.7 mg/kg body weight of a pituitary extract administered 8 h apart [8]. However, the effect of this procedure on health status of the recipient fish has not been studied till now.

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The aim of the present study was to clarify the impact of using Carp pituitary extract (CPE) on some parameters of common carp broodstocks (*Cyprinus carpio*) including clincopathological (RBCs, PCV, Hemoglobin, Cortisol, Glucose, WBCs, Total protein, Albumin, Globulin and A/G ratio), liver and kidney functions tests as well as immunological (nitric oxide production and lysozyme activity). In addition, the histopathological alterations of treated *Cyprinus carpio* broodstocks were documented.

MATERIALS AND METHODS

Fish and Experimental Design: In the present study, Experiment was carried out during April 2014 on 2.5-3 years old ten female common carp (*C. carpio*) broodstocks ranging between 2.5-4 kg body weights as recommended by Brzuska [9], at the hatchery of central laboratory of Aquaculture Research in Abbassa, Sharkia, Egypt. The fish were kept under natural day light and supplied with commercial pelleted ration at a rate of 1% of the body weight twice daily.

Prophylactic measurements were carried out for all fish to avoid parasitic and microbial infection. The fishes were divided into 2 equal groups; each contained 5 fish, which were located in fiberglass tanks as follows:

Group I: Control group, kept under the same circumstances and without injection.

Group II: treated group, intramuscularly injected with two successive doses of CPE. The preliminary dose was 0.3 mg/kg body weight, followed by inductive dose of 2.7 mg/kg body weight, with 8 hours intervals according to the method described by Amer *et al.* [10].

Sampling: Blood samples were taken by syringe from the caudal vessels after 8 hours from the second intramuscular injection. Before sampling, the fish were anesthetized using Quinaldine with a dose of 1ml/40L water bath to prevent struggling according to Bowser [11]. Samples of whole blood were used for determination of blood parameters as RBCs count, WBCs count, PCV and Hemoglobin. Samples of serum were achieved by allowing the blood to clot in refrigerator at 5 °C for 1 h. The clot was centrifuged at 4000 rpm for 15 min to separate the serum for determination of the liver function (AST and ALT), kidney function (SC, BUN), cortisol, glucose, total protein, albumin and globulin.

Clincopathological Analysis: Hemoglobin concentration (g/dl) was determined using the cyanomet-hemoglobin method according to Stoskopf [12]. Packed cell volume (PCV %) was calculated by the microhaemotocritte method described by Decie and Lewis [13]. Erythrocyte and Leukocyte Count was determined using hemocytometer counting chamber and Natt-herrik solution according to Stoskopf [12].

Serum Biochemical Analysis: Serum total protein was determined by Biuret method using stanbio total protein liquid color kit, according to Weichselbaum [14]. Serum albumin was determined according to Dumas and Biggs [15]. Serum globulin was calculated by mathematical subtraction of albumin value from total proteins. Albumin/ Globulin (A/G) ratio was calculated from albumin present in serum in relation to the amount of globulin.

Stanbio kit was used for colorimetric measurement of glucose while monobind cortisol EIA detection kit used to determine the concentration of cortisol in fish serum.

Serum Aspartate amino transferase (AST) and Alanine amino transferase (ALT) activities were estimated calorimetrically using Stanbio kit as described by Reitman and Frankel [16].

Serum Creatinine was measured by the calorimetric method described by Fabiny and Eringhausen [17]. Serum blood urea nitrogen (BUN) was carried out according to the Bertholot reaction described by Fawcett and Scott [18].

Estimation of Some Immunological Parameters: Nitric oxide assay was carried according to method described by Divyagnaneswari *et al.* [19] as follow: Fifty µl of serum was added on an equal volume of Griess reagent in flat-bottomed 96-well plate, followed with gentle shaking. The plate was covered with aluminum foil for 15 min at room temp and then read with ELISA reader at wave length 570. The nitrite concentration was calculated by using Na-nitrite standard curve.

Lysozyme activity was determined according to method described by Esteban *et al.* [20] as follow: 25 µl serum was added on 175 µl (0.75 mg/ml *Micrococcus lysodeikitcus)* in the assay buffer in flat-bottomed 96-well plates. The reduction in absorbance at 450 nm was measured from 0 to 15 min. at 25^{5}C in ELISA reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.00 min^{-1} and the units of lysozyme activity were calculated by using the hen egg white lysozyme standard curve.

Histopathological Examination: For histopathological studies, tissue specimens were obtained from gill, kidney, liver, spleen, skeletal muscle, skin and ovary of carp fish in the two groups and fixed in 10% formol saline for twenty four hours. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained by Hematoxylin and Eosin (H and E) stain for routine examination through the light electric microscope according to Banchroft *et al.* [21].

Statistical Analysis: Analysis of data obtained was performed using Statistical package for the social sciences (SPSS16) computer program.

RESULTS

Clincopathological Findings: The results of haemogram revealed no significant difference in RBCs count, HB value, PCV% and WBCs count among treated group in comparison with control group (Table 1).

The results of protein profile which include Total protein, Albumin, Globulin and A/G ratio showed significant difference among treated group compared with control group only in albumin as revealed in Table 2.

The Liver function (AST and ALT) exhibited no significant difference while serum creatinine exhibited no significant difference between the two studied groups. On the other hand, blood urea nitrogen (BUN) showed statistical significant increase in BUN versus to control group (Table 3). Cortisol and glucose level revealed significant difference among treated group in comparison with control groups as demonstrated in (Table 4).

Immunological Findings: The results of immunological parameters including nitric oxide production and lysozyme activity revealed no significant difference between treated and control group (Table 5).

Table 1: The mean values of total erythrocytes count, packed cell volume, hemoglobin and total leukocytes count

Parameters ----------				
Fish group	RBC x10^6/mm^3	PCV%	HB (g/100ml)	WBC x10^3/mm^3
Control	1.86±0.06	26.45±0.76	8.88±0.22	38.88±0.43
Treated	1.84±0.07	25.30±0.88	8.00±0.42	38.80±0.97

Data are represented as means of five samples ± SE. SE=standard error of mean. P<0.05

Table 2: The mean values of serum protein profile including total protein, albumin, globulin and A/G ratio

Parameters Fish group	Total protein (g/100 ml)	Albumin (g/100 ml)	Globulin (g/100 ml)	A/G ratio
Control	2.74±0.19	1.80±0.13	0.94±0.14	2.07±0.28
Treated	4.16±0.27	3.08±0.31*	1.08±0.11	2.98±0.4

Data are represented as means of five samples ± SE. SE=standard error of mean.

*Significant difference at P < 0.05

Table 3: The mean values of serum amino transferase (liver enzymes), serum creatinine and blood urea nitrogen (Kidney function test).

Parameters Fish group	AST (u/l)	ALT (u/l)	Serum Creatinine (mg/10 ml)	BUN (mg/100ml)
Control	61.60±3.33	43.40±3.3	0.49±0.05	3.12±0.27
Treated	67.40±5.46	51.00±3.39	0.65±0.07	5.00±0.29*

Data are represented as means of five samples ± SE. SE=standard error of mean.

*Significant difference at P < 0.05

Table 4: The mean values of glucose level and cortisol level among control and treated groups.

Parameters Fish group	Glucose level(mg/dl)	Cortisol level (ng/ml)
Control	89.00±1.87	5.74±0.09
Treated	185.00±2.24*	10.46±0.25*

Data are represented as means of five samples ± SE. SE=standard error of mean.

*Significant difference at P < 0.05

Table 5: The mean values of immunological analysis including both lysozyme activity and nitric oxide

Parameters Fish group	Lysozyme activity	Nitric oxide
Control	24.23±3.07	24.38±1.44
Treated	18.94±3.64	27.40±0.86

Data are represented as means of five samples ± SE. SE=standard error of mean. P < 0.05

Histopathological Findings: The kidney revealed dilatation in the blood vessels associated with appearance of homogenous eosinophilic material and blood cells in the lumen of some flattened lining epithelium tubules as well as Focal leucocytes inflammatory cells aggregation was noticed in between the tubules of injected fish with CPE as shown in (Fig.1) and (Fig.2), respectively.

Activation of the ovary was noticed in different stages of vitellogenic follicles of injected fish with CPE as recorded in (Fig.3).

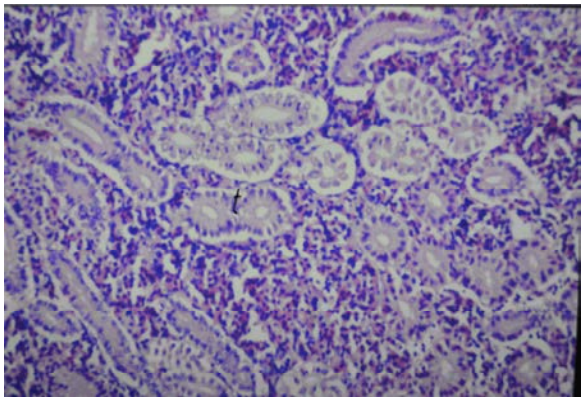

Fig. 1: The kidney revealed dilatation in the blood vessels associated with appearance of homogenous eosinophilic material and blood cells in the lumen of some flattened lining epithelium tubules.

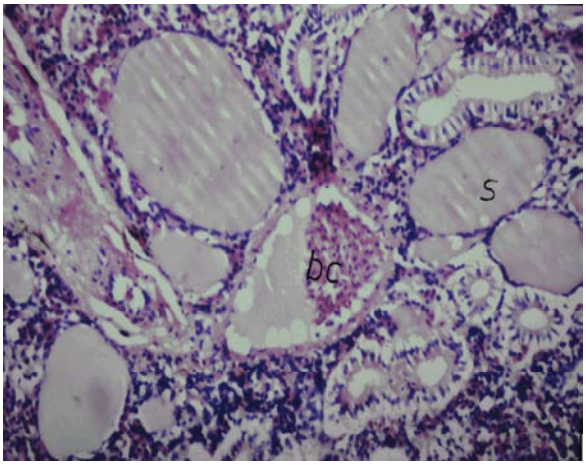

Fig. 2: Focal leucocytes inflammatory cells aggregation in between the tubules.

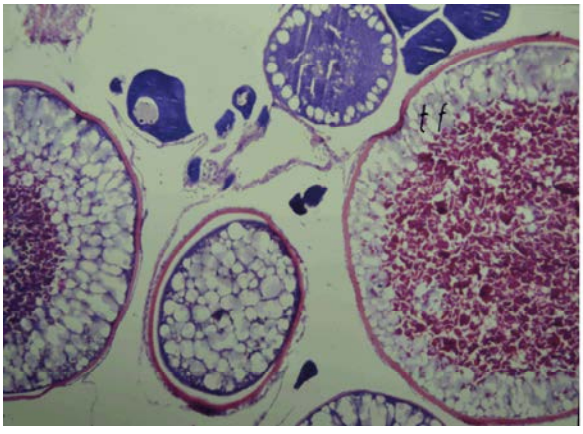

Fig. 3: Activation in different stages of vitellogenic follicles in ovary.

DISCUSSION

Fish reproduction is naturally enforced by a number of environmental factors that consequently controlling reproductive hormones until the release of eggs and sperms. This can also be induced by the injection of external hormones or other substances [22]. One of the most commonly applied spawning agents is carp pituitary extract [23]. Injected pituitary material evades the brain-pituitary link by acting directly on the ovaries and testes, leading to the surge in blood GnRH levels that normally precede spawning [22]. Although the Carp pituitary injection was one of the earliest methods of ovulation induction and spermiation in fish, it is still the most preferable method utilized by many fish culturists. One of the most aggravated stressful circumstances is fish handling during the spawning season. However, the impacts of using pituitary extracts on the health status of broodstock fish have not been studied till now.

Under normal condition, RBCs count, HB value, PCV% and WBCs count were reflected inconsistency during different seasons of common carp. The highest elevated counts of these parameters were achieved in spawning season. Blood cells counts can be used to identify and assess conditions that cause stress to the fish. Their changes may reflect variations in environmental and feeding behavior [24]. Blood plasma is influenced by other factors such as management [25], diseases [26] and stress [27]. Blood serum biochemistry parameters are often used to assess the health status and stress indicators in fishes [28].

In this study, the comparative counts of these parameters between treated and non-treated group revealed no significant differences. One possible reason of these results is that CPE has no adverse effect on hematopoietic tissues and consequently has no stress on fish. These results are in agreement with Kouril *et al*. [29] who found that no differences in plasma indices were recorded between the two groups of female's tench, *T. tinca* treated with carp pituitary and control group.

Serum proteins play an important role in transport of exogenous chemicals and endogenous metabolites [30, 31]. Significant increase in total proteins especially in serum albumin was recorded in CPE treated group; it was two folds higher than the value of control group. These results were consistence with Baker [30] and Mousavi and Yousefian [31] who explained that, Induction of albumin synthesis in spawning time plays an important role in transportation of various components and sex hormones that need for gonads maturation and

egg development. No significant differences in serum globulin and liver enzymes of both groups and the histopathological findings of the liver tissues were apparently normal. The other serum biochemical parameters that have been estimated were cortisol, glucose, blood urea nitrogen and creatinine. Serum samples of the CPE treated group revealed significant increase in cortisol, glucose and blood urea nitrogen levels and non-significant difference was detected in serum creatinine, respect to the control group. This could be attributed to the acute stress exerted upon fish during handling and injection with spawning hormone. In response to this stressful event, the hypothalamic portion of the brain stimulates the release of adrenocorticotropic hormone (ACTH) which activates the internal cells within the head kidney to produce cortisol and other corticosteroid hormones [31, 32]. Cortisol in teleost is a major stress related hormone and it's a primary indicator to stress. Subsequent metabolic pathways arise followed the high cortisol level. It acts as hyperglycemic and catabolic hormone. It activates the glycogen breakdown (glycogenolysis) and protein catabolism (gluconeogenesis) to produce enough energy [33]. This is the main reason accountable for high glucose and blood urea nitrogen in serum of CPE treated broodstocks. An increase in cortisol and glucose levels was also observed by Kime and Dolben [34] during ovulation induced by carp pituitary extract in *C. Carpio*. The high levels of albumin and blood urea nitrogen could be responsible for the mild pathological alterations that have been observed in kidney of the treated groups. The normal and similar values of serum creatinine indicated the kidneys of both groups weren't affected.

Reports on modulation of CPE on the fish immune system are rare. Since the endocrine system of vertebrates is highly interrelated with the immune system [35], two immunological parameters have been investigated in this study; Lysozyme activity and nitric oxide production. Lysozyme is a bactericidal enzyme, involved in hydrolysis of the β (1-4) linked glycoside bonds of bacterial cell wall peptidoglycans. It has been detected in the blood, mucus and organs of various fishes and in oocystes, fertilized eggs and larval stages of several fish species [32, 36]. Lysozyme activity in sera of injected female carp was numerically lesser than control group. However this reduction was statistically insignificant. The gentle reduction in lysozyme activity could be owed to the negative impact of cortisol on the immune system. Wedemeyer [37] stated that high plasma cortisol levels cause immunosuppression and increase the susceptibility of fish to infectious diseases. So it is important to incorporate any type of immunostimulants during the time of spawning to improve the immunological status of fish [35]. The second destructive metabolic product is nitric oxide, it expressed in activated macrophages after microbial infection or injection with foreign materials. The results of nitric oxide production were similar in both groups. The non-significant differences of the innate immunological parameters between groups prove the natural and less immunogenic properties of CPE, derived from the same species of fish. In conclusion, the present study shows that the injections of CPE to common carp broodstocks elevated levels of albumin, blood urea nitrogen, cortisol and glucose significantly difference at ($P<0.05$) after injection with CPE. The hormonal treatment used in the current study was effective in spawning induction of female common carp broodstocks with minimal effect on the health status of treated fish.

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