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Histochemical study of the intestine in the common carp *Cyprinus carpio* fingerlings to detect the impact of replacement of dietary fish and soybean meals with shrimp wastes

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ABSTRACT

The present study investigated the effect of replacement of fish meal and soybean meal with shrimp wastes meal on the intestine tissue in common carp *Cyprinus carpio* fingerlings. Five isonitrogenous (31.66±0.40) and isocaloric (405.57±5.35 kg / 100 g) diets were formulated including control diet (C), 25% and 50% of the total dietary protein replacement of fish meal (T1 and T2 respectively) or soybean meal (T3 and T4 respectively) with shrimp waste meal. Fish were fed with a formulated diet for 63 days. The histochemical study showed increasing in the length of villi and the number of goblet cells, and appearance necrosis in intestine layers, degeneration, shrinking of supranuclear vacuoles, detachment of base of villi, and infiltration of inflammatory eosinophilic granulocytes. Histochemical examination using alcian blue and periodic acid–Schiff revealed three types of specific goblet cells in the middle intestine which secrete neutral mucus, acidic mucus, and a mixture of acidic and neutral mucus depending on their functions. This study argued at the first time effect of formulated diet on intestine tissue histochemically.

INTRODUCTION

Fish meal is an adequate source of protein in aquaculture diets, because its nutritional properties generally meet the nutritional requirements of farmed aquatic organisms **(Lovell, 1989; Hardy, 2010)**.

Many researchers have illustrated the effect of vegetal proteins on the intestine , **Hedrera *et al*.(2013)** mentioned that soybean meal can caused intestinal inflammation process when it used in zebrafish diet. In addition, some experiments discovered side effects of plant proteins on digestive canal in certain fish species , for example it was found that Plant proteins have another negative effects on the structural and functional of the gastrointestinal tract, and they attributed to the presence of anti-nutritional factors **(Francis *et al*., 2001).**

Shrimp waste meal is basically dried waste of the shrimp industry. It is composed of heads, appendages and exoskeleton that is particularly rich in lysine **(Fanimo *et al*., 1996)**. Shrimp waste meal can be used an alternative source of protein for animal

feeding due to its low cost , and it has sustainable value instead of rejecting this waste to the environment **(Souto *et al*., 2018)**.

Histological study of the digestive system is considered a good indicator for nutritional condition of fish; liver and intestine are most important organs in digestion, absorption and metabolism of nutrients **(Rašković *et al*., 2011; Saraiva *et al*., 2016)**. The middle part of intestine is the largest and longest of the gastrointestinal tract which is the main area involved in the digestive processes and the absorption of digested food **(Banan Khojateh, 2012)**.

Histochemical examinations of digestive system have been widely studied in several teleost fish species **(Fiertak & Kilarski, 2002; Domeneghini *et al*., 2005)**. Moreover, histochemical studies are valid appliance for determining the type of cell content, also permit to detect cell damage even in mild signs **(Sirri *et al*., 2017)**.

The current study was carried out to investigate the effect of replacement fish meal and soybean meal with shrimp waste meal on intestine in common carp *Cyprinus carpio* fingerlings using different levels of replacement .This study applied new methodology for discovering the intestinal defects caused by diets replacement using histochemical stains.

MATERIALS AND METHODS

Fish collection and experimental processes

During November 2019, fingerlings of the common carp *Cyprinus carpio* were obtained from fish culture ponds in the Al-Hartha Station of Agricultural Researches, North Basrah at Al-Hartha district affiliated to the Aquaculture Unit, College of Agriculture, University of Basrah. 150 fingerlings with initial mean weights of 11.91±5.77 g were used, they were randomly distributed into 15 experimental glass aquaria(10 fishes each) with dimensions of length 30cm, width 40cm, height 60 cm and constant volume (50 liters) of water. Five food treatments were used, three replications each. Fishes were fed at ratio 3% of body weight twice daily (8:00 am and 1:00 pm) for 63 days. Fishes were weighted biweekly and feed ration was adjusted accordingly. Aquaria were maintained daily by cleaning and siphoning to remove accumulated waste, and half amount of water was approximately replaced for each tank. Water quality parameters of temperature (25±0.92°C), salinity (2.7±0.31 ‰), pH (6.8±0.46) and dissolved oxygen (5.91±0.88) were measured during experimental period. For experimental purposes , all fish were terminated after narcosis with tricaine methanesulfonate solution (0.004%).

Shrimp waste and experimental diets

The shrimp *Metapenaeus affinis* waste were collected from a local fishes market in Basrah city, Southern of Iraq. All shrimp wastes (head, tail and shells) were dried by exposing them to indirect sunlight ,and flipping them daily until became dry, then they

were grounded using electric grinder, sieved through a 0.4 mm mesh size for homogenization and packaged into marked plastic bags. The experimental diets were analyzed the moisture content، crude protein, fat, fiber and ash using Near Infrared Analyzer (Model IL 600).

Five isonitrogenous (31.66 ± 0.40) and isocaloric (405.57 ± 5.35 kg / 100 g) diets were formulated, control diet (C), 25% and 50% of the total dietary protein replacement of fish meal (T1 and T2) or soybean meal (T3 and T4) with shrimp waste meal (Table 1).

Table 1: Ingredients and composition of the experimental diets.

	Experimental diets				
	C	T1	T2	T3	T4
Ingredients					
Fish meal (%)	20.0	15.0	10.0	20.0	20.0
Soybean meal (%)	30.0	30.0	30.0	22.0	15.0
Shrimp waste meal (%)	0.0	8.0	16.0	7.5.0	15.0
Corn meal (%)	15.0	14.0	13.0	15.0	15.0
Barley meal (%)	15.0	14.0	13.0	15.0	15.0
Wheat bran meal (%)	15.0	14.0	13.0	15.0	15.0
Vitamin-mineral premix (%)	3.0	3.0	3.0	3.0	3.0
Binder (CMC)	2.0	2.0	2.0	2.0	2.0
Analyzed composition (%)					
Moisture (%)	8.40	8.23	8.07	8.32	8.24
Crude protein (%)	31.13	31.68	32.22	31.46	31.79
Crude lipid (%)	2.97	4.11	5.24	4.32	5.66
Carbohydrate (%)	48.73	47.17	45.62	46.66	44.60
Ash (%)	6.77	6.81	6.85	7.24	7.71

Histochemical study using Alcian blue and periodic acid–Schiff's staining (AB-PAS).

Intestine samples were fixed in 10% formalin for 48 hours. After fixation, samples were sliced to 0.5 cm thickness and placed in plastic cassettes for dehydration using an automated tissue processor (Histo-Line ATP700, Italy). Samples were embedded with paraffin wax using tissue embedding system (HESTION TEC2800-C, China). The samples were then trimmed and sectioned at 4 μm thickness by semiautomatic microtome (Histo-Line MRS3500, Italy). After that tissue sections were placed carefully in water bath (FALC BI, Italy) and mounted on glass slides using a hot plate (K&K HYSH11, Korea).

Deparaffinised process curried out through two changes of xylene two minutes each, and rehydrated through three changes of different series of ethanol dilutions (100%, 90% and 70%) two minutes each. The tissue sections were then further rinsed in tap water and stained with alcian blue stain (pH 2.5) for 30 minutes then washed with tap water for two minutes. Samples were rinsed in distilled water, immersed in periodic acid for 15 minutes, and washed well in distilled water. Then they were stained with periodic acid–Schiff's reagent for one hour and rinsed in running tap water, then

samples were further rinsed in tap water and stained with Harris's haematoxylin for five minutes and washed in tap water for two minutes. For tissues differentiation, they were immersed in 1% Acid Alcohol Solution for twenty seconds to remove the excessive haematoxylin stain then they washed in tap water for one minute. After that, sections were immersed in 0.2% ammonia water solution for bluing haematoxylin stain for one minute, and washed in running tap water for five minutes. Sections were dehydrated via three series ethanol dilutions (70%, 90% and 100%) for two minutes each, then they cleared using two changes of xylene for two minutes each. Sections were mounted with DPX mounting media and covered by glass coverslip. Images were photographed using a Leica DM1000 microscope.

RESULTS AND DISCUSSION

The histochemical results of current study showed presence number of goblet cells that stained by alcian blue and periodic acid–Schiff (AB&PAS).It is clear that the stains labelled glycoprotein , and the results indicate to a clear difference in the quality of mucus secreted by the goblet cells. Our results agreed with **Neuhaus *et al*. (2007)**; **Marel(2012)** who stated that Glycoproteins interact with alcian blue and periodic acid–Schiff , this reaction labelled neutral glycoproteins, acidic glycoproteins, and neutral-acidic glycoproteins.

Neutral mucus that secreted from the goblet cells participates in the protection of mucosal folding against proteolytic degradation **(Díaz *et al*., 2003)**. It has also a role in the absorption process **(Micale *et al*., 2008)**. Meanwhile, acidic mucus play a crucial role in the protection of digestive canal against bacteria and viruses **(Cohen *et al*., 2016).**

Our findings revealed that there were presence of three types of goblet cells interspersing among enterocytes of the intestine, type I cells were stained purple-red that positive to the PAS stain indicating the presence of neutral mucus, type II were stained blue that positive to the AB stain showing the presence of acidic mucus , and type III cells were labelled blue-purple that positive to the AB and PAS stains indicating to content of a mixture of both acidic and neutral mucus (Fig. 4,7and 10). Our results agreed with the study of koi carp *Cyprinus carpio* var. koi **(Sun *et al*.,2019)**.

The mucin granules that stained by AB and PAS expressed the content of all types of goblet cells (neutral, acidic and neutral and acidic mucin granules) (Fig. 12) . The mucin granules fill the greater part of the goblet cells, they have variable sizes , densities and occupy almost the whole cytoplasm of the cells ,this result is consistent with **Banan Khojasteh *et al*.(2009)**.

Fish fed on control diet (C) displayed necrosis of villi and degeneration of lamina propria (Fig.1), these changes were due to the presence of soybean meal in the control diet. It was detected by several studies that soybean meal in fish dietary causes necrosis of villi and degeneration of lamina propria **(Urán *et al*., 2008 ; Mahmoud *et al*., 2014)**. Whereas, the changes of intestine tissue resulting from feeding common carp on treatment (T1) was represented by necrosis of the circular muscle layer and submucosa and infiltration of inflammatory cells into the areas of necrosis (Fig.2). This finding is similar with study of **El- Hammady *et al*.(2014)**. **Cavalheiro *et al*.(2007)** pointed that shrimp waste meal may be restricted in fish diet due to its high chitin, fiber and ash contents.

The supranuclear vacuoles (SNV) became shrunken and were less regularly aligned, and infiltration eosinophilic granulocytes cells(EGC) have increased (Fig.3) ,this is similar to that described by **Ringo *et al*. (2006)** about krill meal introduced in Atlantic salmon diet . The eosinophilic granulocytes found in fish are similar to those found in mammals, and they have a protective role in fish immunity, as tissue damage causes releasing and translocating these cells to places of damage ,and increasing the number of these cells is evidence of inflammation (Fig.2).

Increasing the percentage of shrimp waste meal in fish diet led to increasing the density of intestinal villi and increasing the number of goblet cells that were observed after feeding on treatment T2 (Fig.5). Furthermore, Fig 6 and 8 have showed necrosis of sub mucosa and villi, degeneration of lamina propria , detachment of base of villi from sub mucosa, and expansion of goblet cells**. Leduc *et al*. (2018)** used diet containing shrimp waste meal in European seabass *Dicentrarchus labrax* feeding , and they had consistent results with current study about intestinal histochemical structure.

Alterations of intestinal histochemical in the fishes fed on the treatment (T3) showed increase of goblet cells, necrosis of villi and degeneration of lamina propria Fig (9), these results consistent with what **Novriadi *et al*. (2017)** stated when they fed Florid pompano fish *Trachinotus carolinus* with 4% of Squid meal in the diet.

The treatment (T4) showed necrosis of villi and longitudinal muscle layer (Fig.10) ,also villi base detachment from circular muscle layer (Fig .11) . Our results confirmed the results that found by **Yadav *et al*. (2014)** on Asian catfish *Clarias batrachus* fed on diets with different levels of fats. In addition, treatment (T4) indicated that fishes fed on diet containing 50% SWM has shown natural mucin granules, natural - acidophilic mucin granules, and acidophilic mucin granules in goblet cells at bottom of villi (Fig.12).

In conclusion , we have shown that shrimp waste meal in formula diet has noticeable effect on the structure of intestine tissue in the common carp *Cyprinus carpio* fingerlings. This study also revealed that there are several damages in the layers of intestine, and there are three types of mucous cells in the intestine tested by specific

histochemical markers (alcian blue and periodic acid–Schiff) .More studies are demanded in the future in field of dietary influences using immunohistochemistry or fluorescent immunohistochemistry.

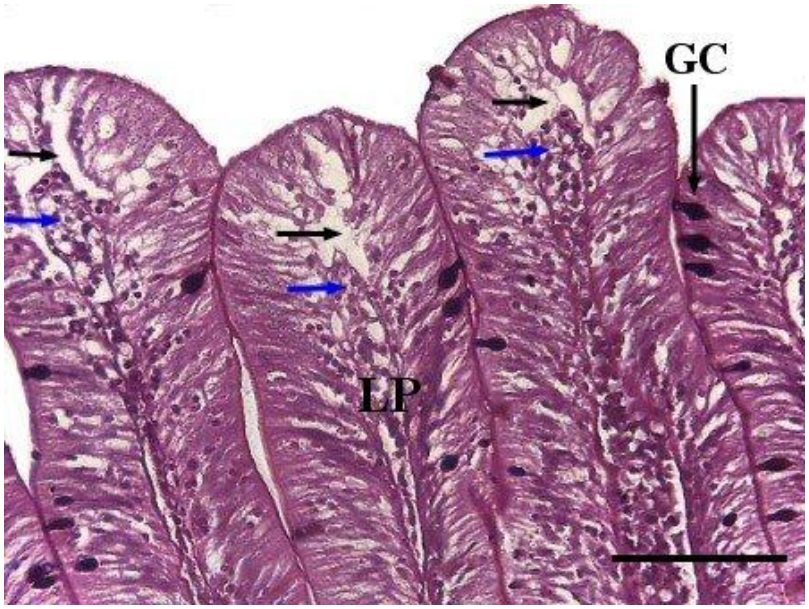

Fig. (1): A cross section in the intestine of the common carp *Cyprinus carpio*, showing the effect of treatment (C). (GC), Goblet cell ; (LP) ,degeneration of lamina propria (blue arrow) ; necrosis of villi (black arrow). Alcian blue and periodic acid–Schiff's staining (AB&PAS). Scale bar= 50µm.

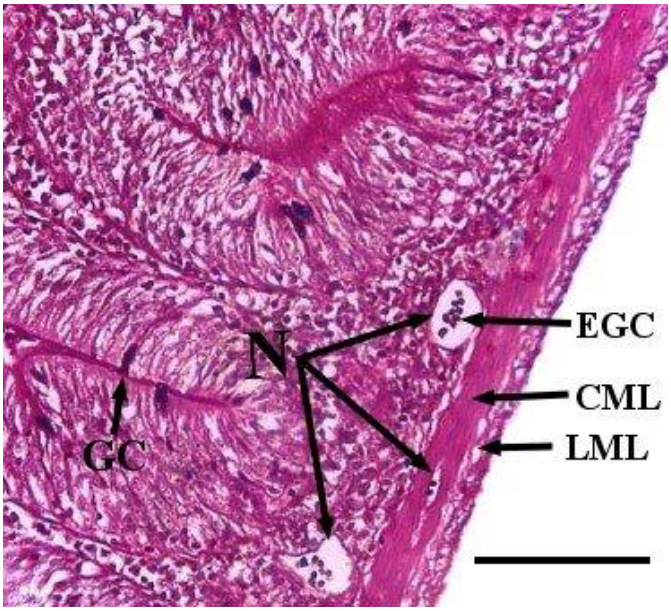

Fig. (2): A cross section in the intestine of the common carp *Cyprinus carpio*, showing the effect of treatment (T1). (N), necrosis of sub mucosa and circular muscle layer (CML); (EGC), eosinophilic granulocytes ; (GC), goblet cell; (LML), longitudinal muscle layer. Alcian blue and periodic acid–Schiff's staining (AB&PAS) . Scale bar= 50µm.

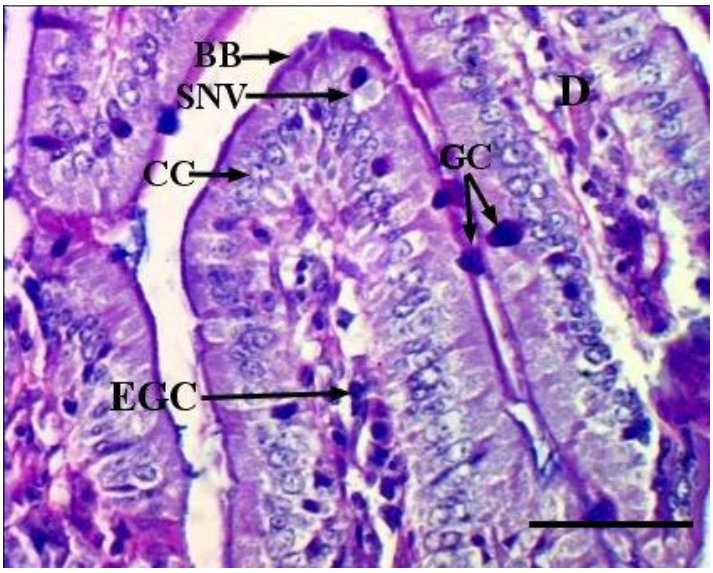

Fig. (3):A cross section in the intestine of the common carp *Cyprinus carpio*, showing the effect of treatment (T1). (D), degeneration of Lamina propria (LP); shrinking of supranuclear vacuoles (SNV); (BB), Brush border; (CC), Columnar cells; (EGC), eosinophilic granulocytes; (GC), goblet cell. Alcian blue and periodic acid–Schiff's staining (AB&PAS).Scale bar= 50µm.

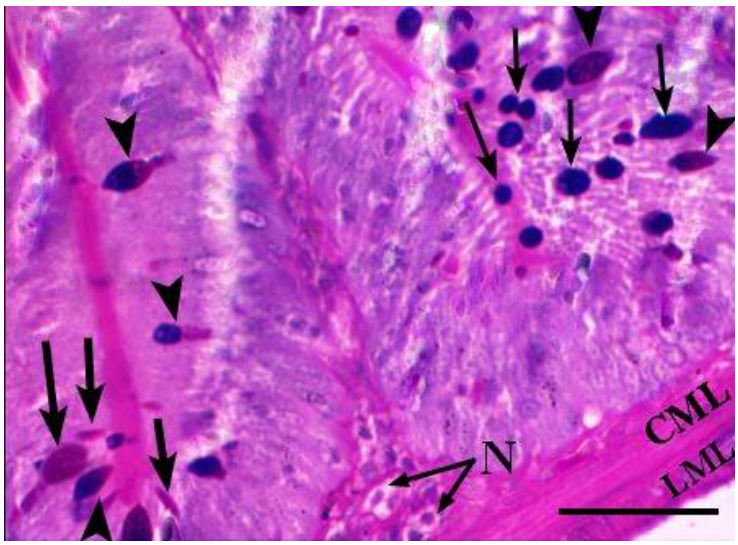

Fig. (4):A cross section in the intestine of the common carp *Cyprinus carpio*, showing the effect of treatment (T1), and distribution of three types of goblet cells: type I (thick arrow); type II (thin arrow); and type III (arrow head); (N) necrosis of sub mucosa; (CML),circular muscle layer; (LML) longitudinal muscle layer. Alcian blue and periodic acid–Schiff's staining (AB&PAS).Scale bar= 50µm.

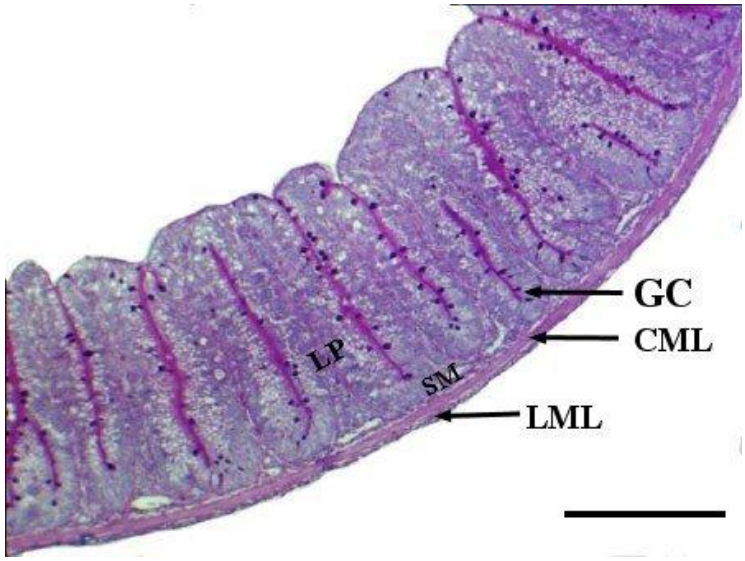

Fig. (5): A cross section in the intestine of the common carp *Cyprinus carpio* in treatment 2 (T2) showing the increase of the intestine villi , and goblet cells number (GC); (CML), circular muscle layer ; (LML), longitudinal muscle layer ; (LP),Lamina propria; (SM), sub mucosa. Alcian blue and periodic acid–Schiff's staining (AB&PAS).Scale bar= 200µm.

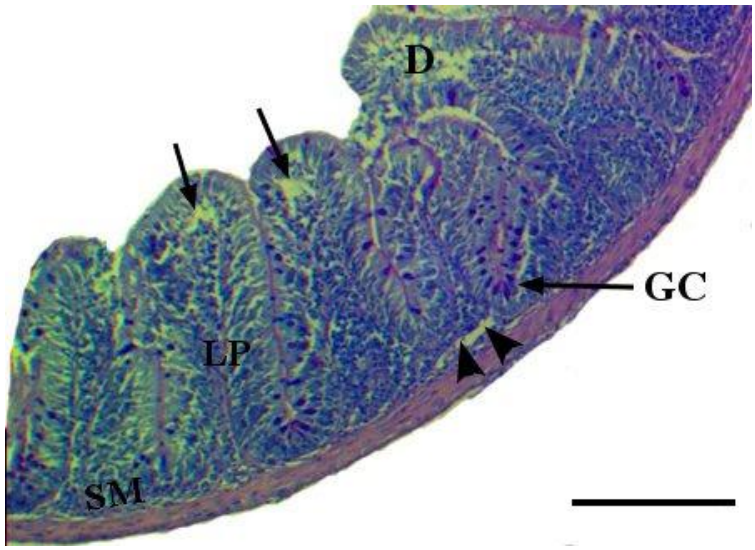

Fig. (6): A cross section in the intestine of the common carp *Cyprinus carpio*, showing the effect of treatment (T2). Necrosis of villi (black arrow);(D), degeneration of lamina propria (LP); detachment of base of villi from sub mucosa (arrow head);(GC), goblet cell. Alcian blue and periodic acid–Schiff's staining (AB&PAS) .Scale bar= 200µm.

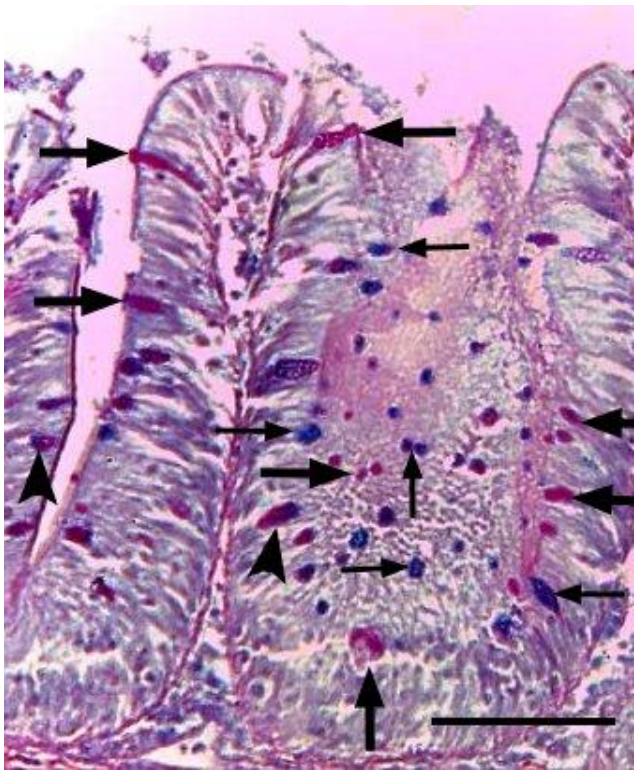

Fig. (7): A cross section in the intestine of the common carp *Cyprinus carpio*, showing the effect of treatment (T2), and distribution of three types of goblet cells, type I (thick arrow); type II ; (thin arrow); type III (arrowhead). Alcian blue and periodic acid–Schiff's staining (AB&PAS). Scale bar= 50µm.

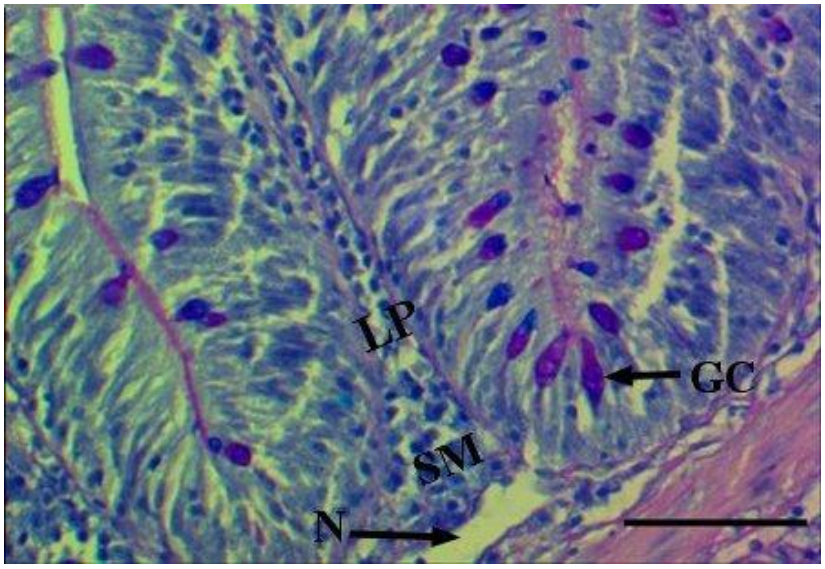

Fig. (8):A cross section in the intestine of the common carp *Cyprinus carpio*, showing the effect of treatment (T2). (N) necrosis of sub mucosa (SM); expansion of goblet cells (GC); (LP), lamina propria. Alcian blue and periodic acid–Schiff's staining (AB&PAS).Scale bar= 50µm.

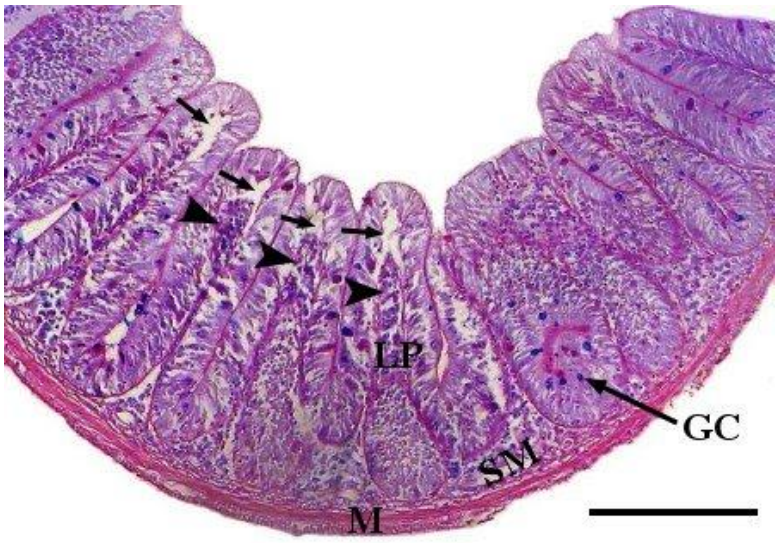

Fig. (9): A cross section in the intestine of the common carp *Cyprinus carpio* in treatment 3 (T3) showing necrosis of villi (black arrow); degeneration of lamina propria (arrowhead); increasing of goblet cells (GC) ; (M), muscularis ; (SM), sub mucosa. Alcian blue and periodic acid–Schiff's staining (AB&PAS).Scale bar= 200µm.

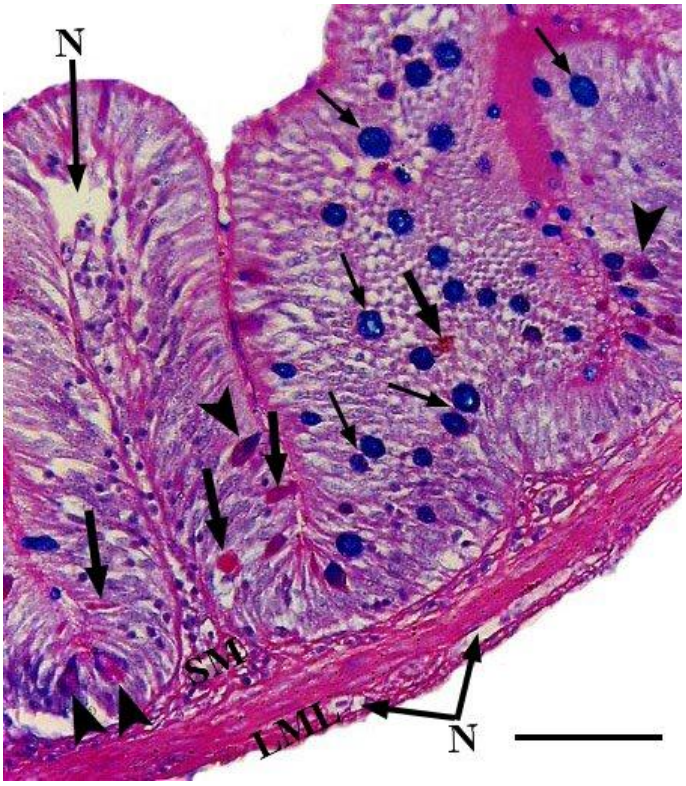

Fig. (10): A cross section in the intestine of the common carp *Cyprinus carpio*, showing the effect of treatment (T4).N, necrosis of villi and longitudinal muscle layer (LML) ; distribution of three types of goblet cells, type I (thick arrow); type II (thin arrow) ; type III (arrow head).(SM), sub mucosa. Alcian blue and periodic acid–Schiff's staining (AB&PAS).Scale bar= 50µm.

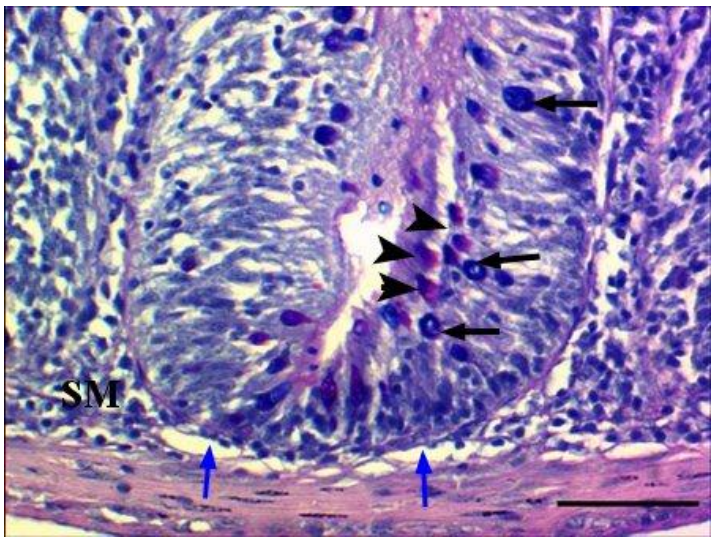

Fig. (11): A cross section in the intestine of the common carp *Cyprinus carpio*, showing the effect of treatment (T4) . Detachment of the villi base from circular muscle layer (blue arrow); type II of goblet cells (black arrow); type III of goblet cells (arrowhead); (SM),sub mucosa. Alcian blue and periodic acid–Schiff's staining (AB&PAS) .Scale bar= 50µm.

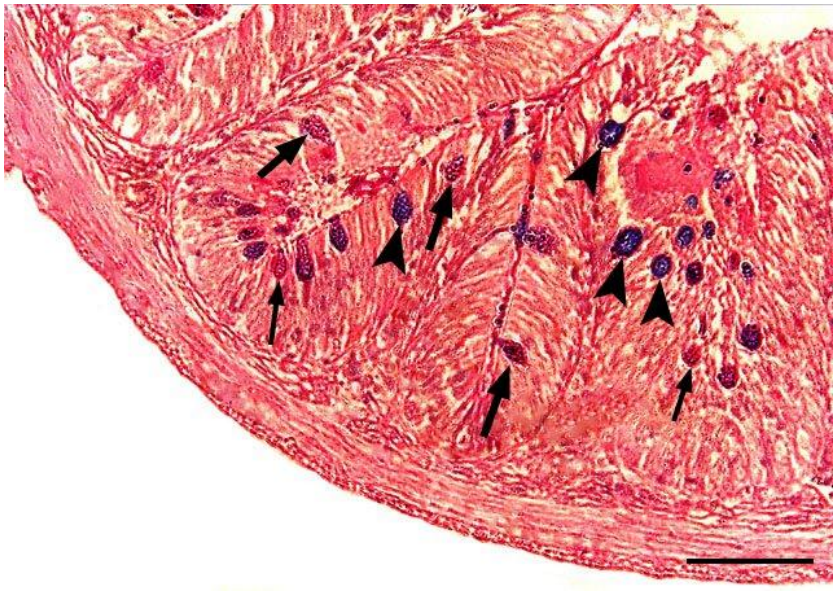

Fig. (12):A cross section in the intestine of the common carp *Cyprinus carpio*, showing the effect of treatment (T4). Natural mucin granules (thin arrow); natural mucin granules and acidophilic mucin granules (thick arrow); and acidophilic mucin granules (arrow head) were observed in goblet cells at bottom of villi. Alcian blue and periodic acid–Schiff's staining (AB&PAS) .Scale bar= 50µm.

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