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Full Length Research Paper

Impact of different levels of dietary myo-inositol on the growth performance, histological structure of gonads and liver of red tilapia reared in brackish water

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A 120 day growth trial was conducted to evaluate the dietary inositol using diets (25.7% crude protein pelleted diet). These diets were formulated to contain graded levels of supplemental myo-inositol (MI) (0, 300, 400 and 500 mg/kg). Four conical bottom fiberglass tanks (500 L) were used and stocked with fifty red tilapia in each tank with initial weight (average weight) of 1.82 g ± 0.03). Fishes were fed twice daily at a rate of 8% of live body weight for 6 days a week. Significantly, the best value of average final body weight was observed at 500 mg/kg inositol (MI) followed by 400 mg/kg diets ($p<0.05$). Female red tilapia which fed on diets that contained 400 and 500 mg/kg inositol showed many types of atresia, each one had its specific feature such as degeneration of the nucleus, liquification of cytoplasm and yolk. Also, hypertrophy of the follicular cell acted as phagocytic cells which invaded the oocyte from out side. The testis of red tilapia fed diets supplemented with 300 and 400 mg/kg was not affected by dietary treatments and the results revealed normal development of active spermatogenesis process at maturation, nearly ripe and spawning stages, while the testis of fish fed diets supplemented with 500 mg/kg showed resorption of spermatozoa and thick lobules boundary tissue. The liver of fish fed diets that contained 300 mg/kg (MI) revealed that hepatocytes had distracted membrane and faintly stained cytoplasm. Pycnotic nuclei are common. With increased dose of (MI) to 400 mg/kg the pyenotic nuclei and cytoplasmic vacuolation was obtained. With increased dose of (MI) to 500 mg/kg diets, lipid infiltration, lipid metabolic disturbance resulted in the accumulation of lipid in liver, decreased hepatic lipoprotein output and intestinal lipodystrophy. The recommended dose is 400 mg/kg diets to obtain normal males. Also, the results explained the mechanism of inositol action through reproduction dysfunctions and recruitment failure which occurred at dose more than 400 mg/kg (MI) and the recruitment caused a reduction in population size.

Key words: Myo-inositol, red tilapia, histological structure, gonads, liver.

INTRODUCTION

The gonads are bi-functional organs that produce germ cells and sex hormones. The ovaries produce sex hormones (estrogens and progestins) and ova. The testes produce testosterone and spermatozoa .Proper functioning of the gonads depends upon structural and hormonal integrity necessary for reproduction and hence survival of species.

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There is no doubt that many environmental parameters (temperature, light, salinity, nutrition, etc) are required for the initiation of complex changes that control the reproduction of teleosts. Any change in these factors will in term affect the external receptors and hence the central nervous system, hypothalamus, pituitary and gonads that affect processes required for steroids biosynthesis within gonads which greatly effect gonadal development, ova and sperm ripening.

Myo-inositol (MI) is the biologically active isomer of inositol, a structural component in living tissue which

Table 1. Percentage of contents and the chemical analysis of pelleted diet

Ingredients	%
Fish meal	20.00
Soybean meal	20.00
Wheat bran	28.00
Starch	20.00
Fish oil	5.00
Corn oil	3.00
Yeast	2.00
Vitamin premix	1.00
Mineral mixture	1.00
Chemical analysis (as DM basis)	
Dry matter	91.84
Crude protein	25.74
Ether Extract	11.97
Crude fiber	55.46
Ash	6.83
Gross energy (Kcal/100 g DM)	486.1
Protein/energy ratio (mg CP/Kcal GE)	52.95

commonly occurs as a component of phospholipids in animal cell membranes. As a part of the phospholipids form, phosphatidylinositol is an important participant in transmembrane signal transfer (Aukema and Holub, 1994). MI is classified as a vitamin-like nutrient and is often supplemented to fish diets for some fish species. The quantitative requirement of inositol for growth has been reported for some fish species including carp (Aoe and Masuda, 1967), Red sea bream (Yone et al., 1971), atlantic salmon (Waagb et al., 1998) and for juvenile hybrid tilapia (Shi-Yan and Shu-Lin Su, 2005). Some fish species including channel cat fish (Burtle and Lovell, 1989) and hybrid striped bass (Deng et al., 2002) have been reported to lack response to dietary supplement of inositol. Fish and other vertebrates may synthesize inositol in their intestinal microbial flora, (Farrag et al., 2008). Although, the physiological role of (MI) is still unclear, it is believed to play an important role in the growth of liver cells , bone marrow cells, liver lipid (cholesterol) transport and in the synthesis of RNA (Shiau and Su,2003).

With the knowledge that some fish species require diets supplemented with inositol and some other species do not require dietary inositol this study was designed to determine the essentiality requirement of (myo-inositol) in red tilapia which consider one of the most important and economical cultured species in Egypt and may be around the world. The effect of (MI) on fish growth performance, gonads maturation from the histological view points as well as on liver was also employed during the present study.

MATERIALS AND METHODS

Place of study, fish stock and performance

Experiments were carried out at the fish rearing Lab, National Institute of Oceanography and Fisheries, Alexandria branch located in El-Anfoushy Egypt. Twelve conical bottom fiberglass tanks (500 L volume each as triplicate) were used during this experiment supplied with brackish water (25.2 ± 0.5°C). Fifty healthy hybrid red tilapia advanced fry were stocked in each tank with initial weight ranged from 1.74 to 1.90 g (average weight 1.82 ± 0.03 g). Red tilapias which were used in the present investigation were free from parasites and bacterial or viral diseases and no pathological alterations were evident in the fish viscera.

Fishes were fed for 120 days (rearing period), twice daily at a rate of 8% of live body weight for 6 days a week with 25.7% crude protein pelleted diet (Table 1) supplemented with myo-inositol in different levels (0, 300, 400, 500 mg/kg): T1, T2, T3, and T4, respectively. At the end of the experiment the final weight was reported.

Growth performance of red tilapia advanced fry were monitored by the measurement of total length and weight of a sample group; about 25 individuals, once a month from each treatment in order to determine growth parameters (Farrage et al., 2003).

Histological Examinations

At the end of the experiment, some organs (ovaries, testis and liver) were fixed in Bouin's fluid and 10% natural buffered formalin, following fixation tissue specimens were dehydrated, cleared and then embedded in paraffin wax at 60°C. Sections of 3 - 8 µm thickness were cut and stained with Harris's hematoxylin and eosin for routine histological examinations.

Statistical analysis

Statistical analysis was carried out by ANOVA and Duncan's test.

RESULTS

Fish performance

Data in Table 2 shows high significant differences ($p < 0.05$) of growth performance due to a dietary supplement of inisitol (MI) in their diets. The highest value of average final body weight was observed in diet with 500 mg/kg (49.33 g) T4 followed by 400 mg/kg (47.33 g) T3. The same trends were obtained for average daily gain and specific growth rate parameters. Furthermore, red tilapia of T3 and T4 treatments possessed also good survival rates of 84 and 88%, respectively.

Histological observations of ovaries

The microscopic examination of the control ripe ovaries of red tilapia revealed the presence of different develop-mental stages of oocytes as shown in Figure 1. This means that the ovaries of red tilapia can be categories as asynchronous ovaries development. From Figure 1, it is clear that consecutive developmental stage of the

Table 2. Means ± SE of final body weight (g), average daily gain (ADG) (mg), specific growth rate (SGR) and survival rate under different (MI) concentrations

Parameter	Concentration of (MI) (mg/Kg)			
	0	300	400	500
Average initial weight (g)±SE	1.82 ±0.03	1.82 ±0.03	1.82 ±0.03	1.82 ±0.03
Average final weight(g))±SE	29.39±1.63^{b}	38.35±4.19^{C}	47.33±1.27^{a}	49.33±3.24^{a}
Average daily gain(ADG)(mg) ±SE	229.75±34.97^{b}	304.41±24.55^{c}	379.25±22.56^{a}	395.91±25.21^{a}
Specific growth rate (SGR) ±SE	2.31±0.02^{b}	2.53±0.09^{a}	2.71±0.07^{a}	2.91±0.021^{a}
Survival rate (%)	92	84	84	88

*In the same row, means having different superscript are significantly different ($p<0.05$).

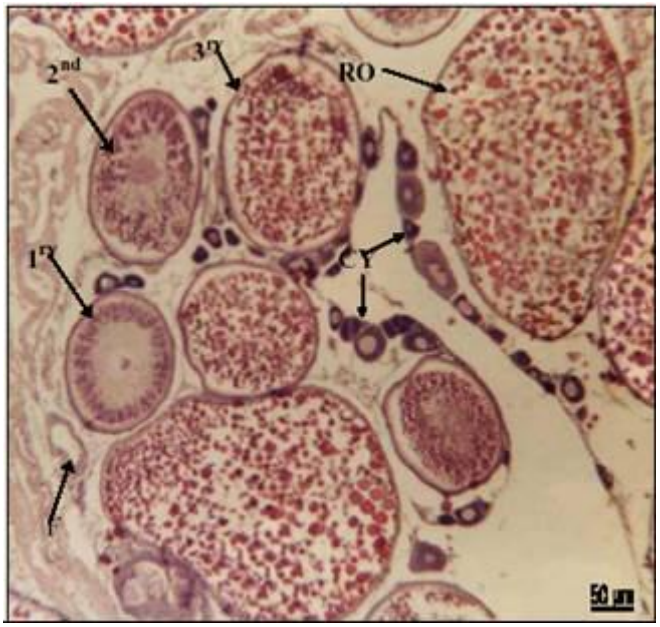

Figure 1. Cross sections of ripe ovary of red tilapia showing: several developmental stages, cytoplasmic growth stage (CY); vitellogenic stage (1ry, 2nd and 3ry), yolk deposition stages; ripe ova (RO) and empty follicle (F) (Bar indicates 50 µm).

cytoplasmic oocyte (immature cell) to mature oocytes (vacuoles and vitellogenic oocyte) developed to ripe ova. Cytoplasmic growth stage was characterized by large nucleus which occupied a great part of the cell that contained numerous nucleoli .The diameter of the oocyte ranged between 25 ± 8 µm and 125 ± 10 µm in average, while the nucleus ranged between 15 ± 5 µm and 60 ± 8 µm in diameter and contained clear chromatin material.

Yolk deposition stage was characterized by the presence of yolk granules which are formed from the peripheral region of the cytoplasm as shown in primary yolk stage (1ry). Further growth of oocyte was accompanied by centrapetal yolk deposition as show in secondary (2ry) and tertiary yolk deposition stage (3ry). The oocyte mean diameter increased from 300 ± 60 × 450 ± 70 µm along its short and long axis for 1ry to 400 ± 40 µm × 600 ± 70 µm in (2ry), respectively. The 3ry and ripe ova increased in diameter ranged between 520 ± 45 × 830 ± 70 µm and 960 ± 63 × 100 ± 130 µm.

The microscopic examination of the ovaries for female fed with diets supplemented with 300 mg/kg (MI) revealed the normal growth phase of immature oocytes (cyto-plasmic growth) and randomly normal growth phase of mature oocytes (vacoulized and vitellogenic stages) Figure 2.

The results show the presence of atretic oocytes for mature oocytes which belong to second atretic category.

At the early stage, the second category showed that the cytoplasm lost its natural appearance and nucleus degenerated as shown in Figures 2b, c and d. The last stage of atrisia in vitellogenic stages can be detected in Figures 2 a, b and 2e showing proliferations of the follicles cell break down of the yolk granules. The central area of the oocyte showed generative process. The yolk was resorbed and there were large number of different vesicle present in the cytoplasm.

In Figure 2f, the nucleus disappeared, the cytoplasm became liquidized, the follicular cell multiplied and hypertrophy acted as phagocytic cell which invaded the oocyte from outside.

The examination of the ovaries of red tilapia fed diets supplemented with 400 and 500 mg/kg (MI), respectively revealed atretic oocyte at vitellogenic stages that showed reduction and deformation of the nucleus and the yolk were pressed in the inter cellular space (Figures 3c and d) which lead to complete yolk resorption and there were large number of different vesicle present in cytoplasm and the nucleus (Figures 3a, b and c and Figures 4 a and c).

Moreover, fish fed diets supplemented with 500 mg/kg (MI) revealed the presence of early yolk vesicle stage in the first step of atresia. The nucleus was deformed and surrounded with large vacuoles (Type A) Figure (4c). Also, the results revealed that the follicle cell of oldest oocyte multiplied and hypertrophy and they acted as phagocytic cell and attacked the oocytes from out side and the nucleus degenerated (Figure 4b). The results revealed increased abnormalities and atretic cell of mature oocyte with increase in the concentration of MI from 400 to 500 mg/kg as shown in Figures 3 and 4. Also the results revealed increased abnormalities and atretic

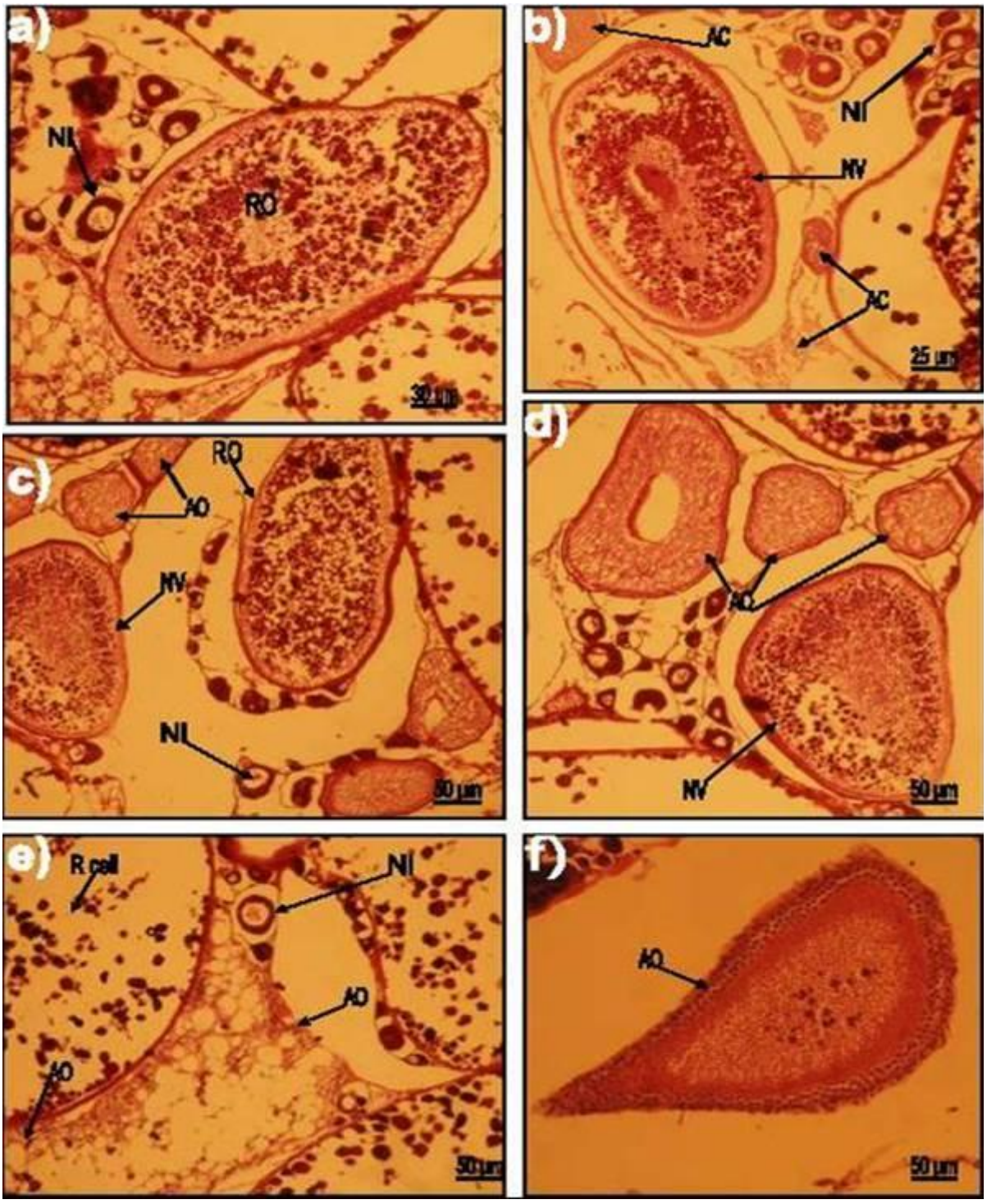

Figure 2. Cross sections of mature ovary of red tilapia fed on diets supplemented with 300 mg/kg of (MI) showing normal ripe ova (RO), normal immature cell (NI) (cytoplasmtic growth) (**a**, **c** and **e)**, normal vitellogenic cell (NV) (tertiary yolk deposition stage) (**b**, **c** and **d)** and atritic cell (AO) (**f)**. (Bar indicates 25, 30 and 50 µm).

cell of mature oocyte (vacuolized and vitellogenic cells stage) with increasing the concentration of (MI) from 400 mg/kg to 500 mg/kg as shown in Figure 3 and 4. Also, it was revealed that increased follicular atresia of both yolk and previlellogenic oocytes, led to abnormal yolk deposition and yolk formation in oocyte. The theca external and some follicular cell could become phagocytes as this type of atresia was observed in the spawning

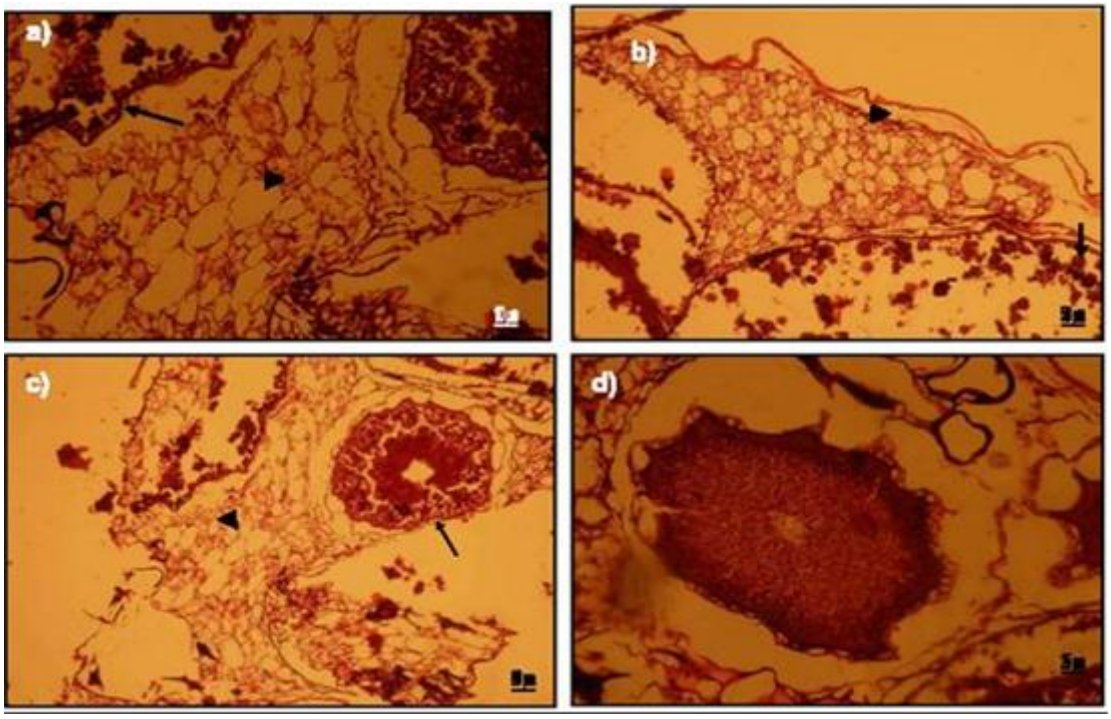

Figure 3. Cross sections in ovary of ripe female red tilapia fed on diets supplemented with 400 mg/kg of (MI) showing: (**a**) Atretic oocyte (head arrow) and disintegrated follicular wall (arrow) (Bar indicates 10 µm), (**b**) disintegrated ova cell (head arrow) and deformed vitellogenic oocyte (arrow) (Barindicates 50 µm), (**c**) atretic oocyte (arrow) and disintegrated ova (head arrow) (Bar indicates 80 µm) and (**d**) atretic ova.

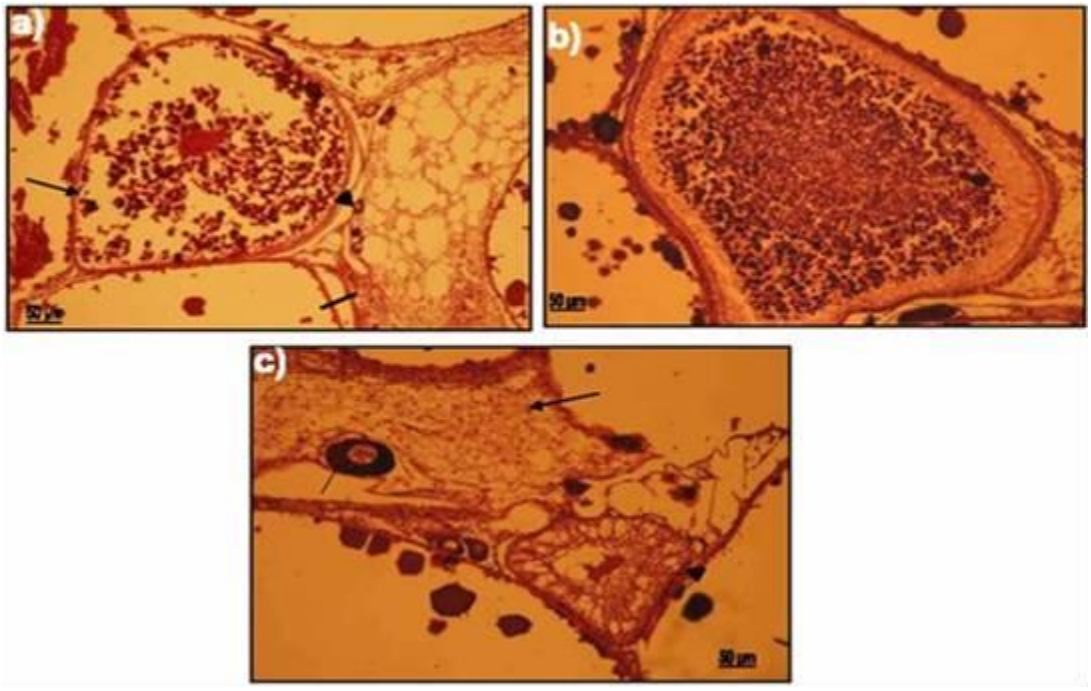

Figure 4. Cross sections of female ripe ovary of red tilapia fed diets supplemented with 500 mg/kg of (MI) showing: (**a**) Disintegrated ova (arrow) and normal immature cell (head arrow), NV normal Vitellogenic oocyte (bar indicates 50 µm), (**b**) Atretic oocyte (bar indicates 50 µm), (**c**) disintegrated oocyte (arrow), normal immature cell (line) and early yolk vesicle stage in first step of atresia (head arrow) (bar indicates 50 µm).

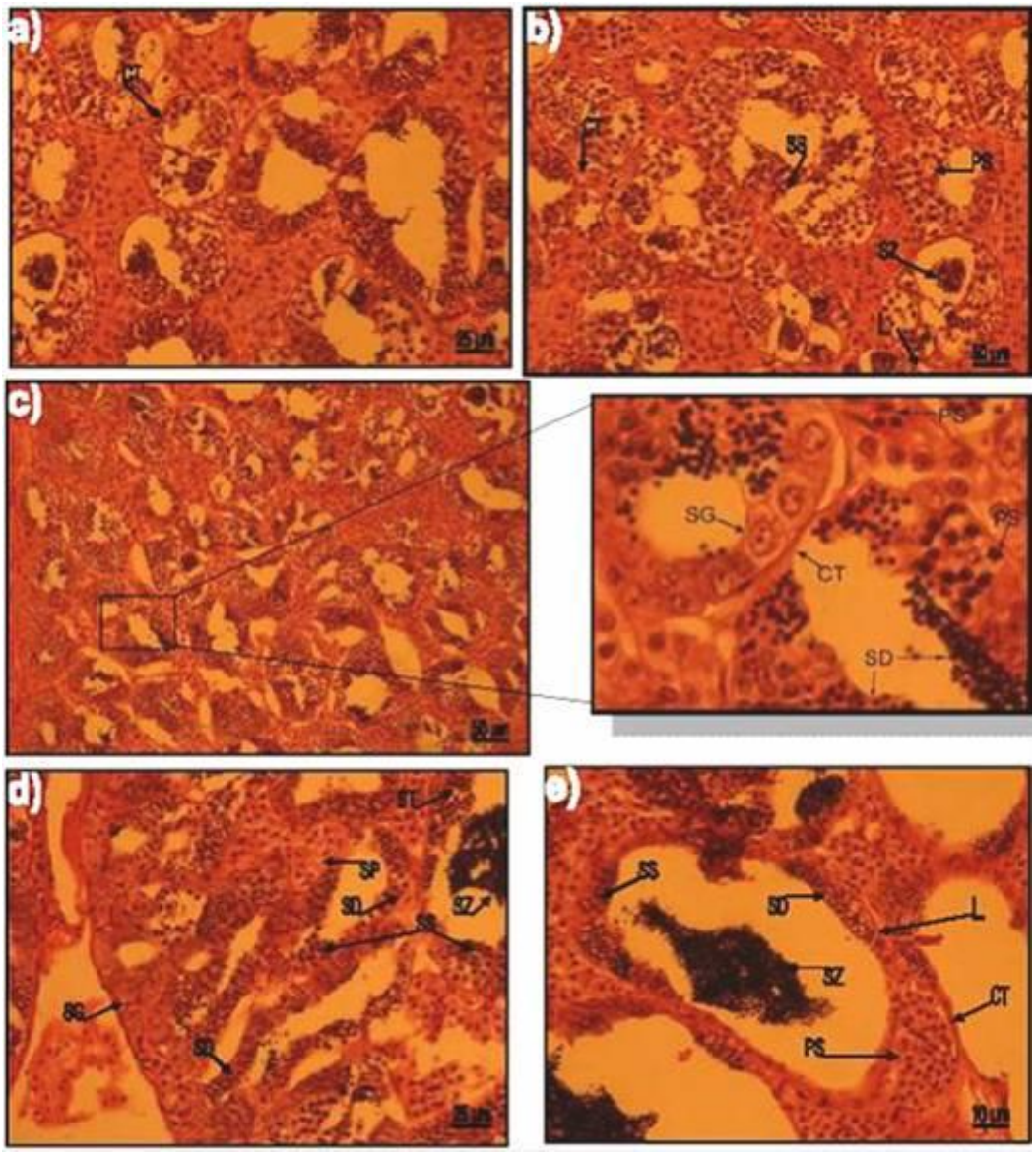

Figure 5. Cross sections of mature testes of red tilapia showing active spermatogenesis, spermatogonium (SG), secondary spermatocytes (SS), spermatide (SD), spermatozoa (SZ) and inter lobular connective tissue (CT), primary spermatocyte (PS) and lobules (L); (**e**) Cross sections of nearly ripe testes showing, spermatogonium (SG), primary spermatocyte (PS), secondary spermatocytes (SS), spermatide (SD), spermatozoa (SZ) and connective tissue (TC) for fish fed on diets supplemental with 300 mg/kg (MI) (Bars indicate 10, 25 and 50 µm).

season and fully mature ovaries.

Testis

The testis of red tilapia was surrounded by testicular wall composed of thin membrane of connective tissue. The testis was characterized by the presence of long branched seminiferous lobules held to each other by interstitial connective tissues.

The seminiferous lobules at mature stage of fish fed diets that contained 300 and 400 mg/kg (MI), had normal various types of cells at different developmental stage; spermatogonia (SG), primary spermatocytes (PS), secondary spermatocytes (SS), spermatides (SD) and few of spermatozoa (SZ) as shown in Figures 5a, b, c and d) for 300 mg/kg (MI) and Figures 6a and b) for 400 mg/kg of (MI). Spermatogonia (SG) was nearly rounded, its diameter ranged from 12 µ to18 µ and the nucleus ranged from 7 µ to 10 µ. A nucleus lies in the periphery and it is located in the center of the same cells (Figure 6 c).

Primary spermatocytes (PS) are produced by mitosis

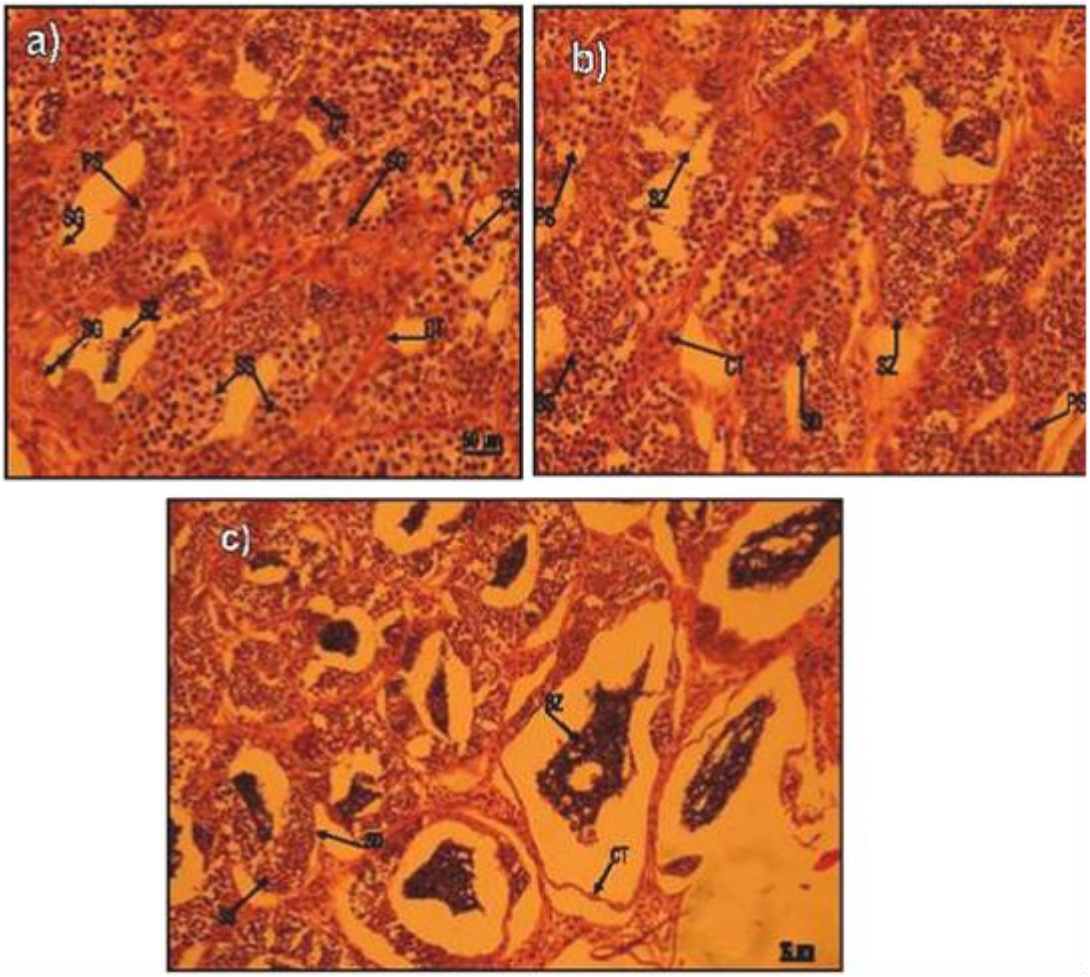

Figure 6. Cross sections of mature testes of red tilapia showing (SG),/(PS),/(SS),/(SZ),/ (CT), and/ (L); **(c)** cross sections of spawning testes showing (SS), (SD), (SZ) and (CT) for fish fed on diets of 400 mg/kg (MI) (Bar indicates 10 and 25 µm.).

division of spermatogia. Its nucleus is smaller than spermatogonia which ranged in size from 4 µ to 5 µ. The PS was slightly polygonal in shape with a pale cytoplasm and a nucleus densely packed with chromatin material which concentrated on one pole of the nucleus (moon shaped arrangement) as shown in Figure 5c.

Secondary spermatocyte (SS), are produced by meiotic division of the PS. They are relatively smaller in size; its diameter measured about 3 µ. Their nucleus occupied most of the cell. It was also very dense and dark, where the chromatin material was thick and deeply stained.

Spermatide (SD) arose as a result of meiotic division of the SS. They measured about 2 µ, with no distinguishable cytoplasm. (Figure 5d).

The nucleus was spherical with dense chromatin material that occupied most of the cell. The spermatide were found in nests. They were found at the end of the maturation stage and tended to be pushed toward the center of the lobule lumen (Figures 5b and e).

Spermatozoa (SZ) are the smallest cell among the male germ cell. They were formed when the spermatides underwent metamorphosis to form the sperms; the nucleus of the spermatide became more dense and formed the head of the spermatozoa which was spherical with diameter of about 2 µ. Spermatozoa are usually concentrated in the central zone of the animal lobules, particularly near the spermatic ducts (Figure 5 e). The testis of fishes fed diets of 400 mg/kg MI was not affected by dietary treatments and they showed normal development of spermatogenesis process at maturity; nearly ripe and spawning stage as shown in Figures 6a, b and c. The testis of fish feed diets that contained 500 mg/kg (MI) showed negative effect and showed resorped spermatozoa and thickened interlobular connective tissue (Figures 7a, b and c).

The liver

The results indicated abnormalities such as hyperplasia,

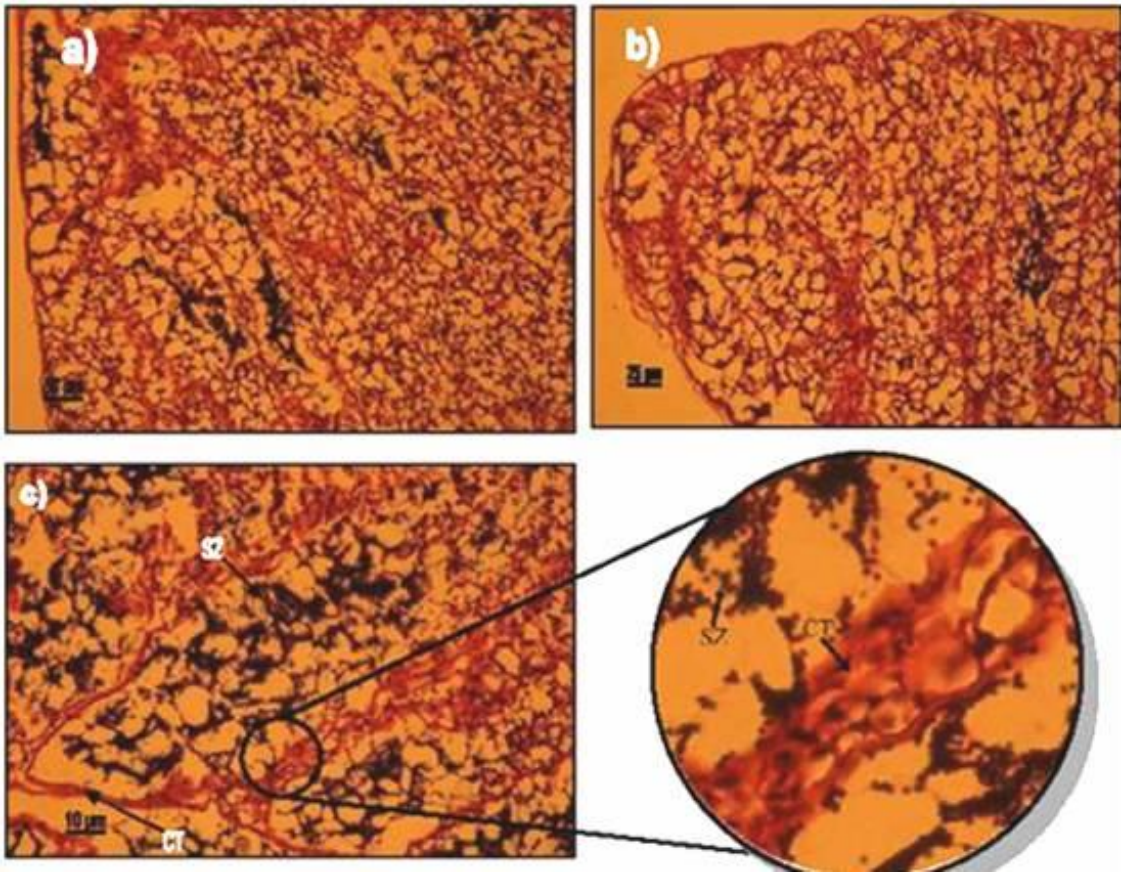

Figure 7. Cross sections in ripe testes of red tilapia fish fed on diets that contained 500 mg/kg of (MI) showing resorped spermatozoa (SZ) and thickened Interlobular connective tissue (CT). Bar indicates 10, 25 and 50 µm.

hypertrophy and degeneration of hepatocytes. The histological changes in liver of red tilapia included necrotic changes with variation in staining intensity, distortion of liver cell orientation, shrinkage of hepatocytes, pycnosis of nuclei, cytoplasmic vacuolation, intensive fatty infiltration due to abnormal lipid accumulation as consequences. The liver of the fish feed diet supplemented with 300 mg/kg (MI) showed that hepatocytes had distracted membrane and faintly stained cytoplasm. Pycnotic nuclei were common. Figures 8a, b and c shows obliteration in normal arrangement of hepatocytes, distortion of liver cell orientation and shrinkage of hepatocytes. The increased dose of (MI) to 400 mg/kg showed the presence of pycnotic nuclei and cytoplasmic vacuolation, while treatment with 500 mg/kg MI increased the intensity of pycnotic nuclei and cytoplasmic vacuolation with intensive fatty infiltration.

DISCUSSION

The results revealed high significant differences on growth. The highest value of average final body weight was observed in group of fish fed with diet of 500 mg MI/kg followed by 400 mg MI/kg. Also, these treatments (T4 and T3) possessed a good survival rates during the study. This may reflect the adequate requirements of Myo-inositol in the enhancement of fish growth and survival.

In fish nutrition, evidence suggests that metabolic synthesis of inositol occurred to some degree in liver kidney and other tissues (Deng et al., 2002).

For some fish species, new synthesis is inadequate to support their metabolic needs and thus require an exogenous source of this vitamin as found in common carp, red sea bream, yellow tail and Japanese eel fed inositol (Aoe and Masuda, 1967; Yone et al., 1971; Arai et al., 1972). The reported quantity require for these species range from 250 to 500 mg/kg diet (NRC, 1993). Some species as channel cat fish, Asia sea bass, sunshine bass and Nile tilapia do not require a diet supplemented with inositol for normal growth and are probably able to synthesize sufficient quantity of inositol for the normal erythropoiesis (Burtle and lovell, 1988; Boonyoratpalin and wanakowat, 1993; Deng et al., 2002; Peres et al., 2004). In the present study, the needed doses of (MI) may be species specific. The results

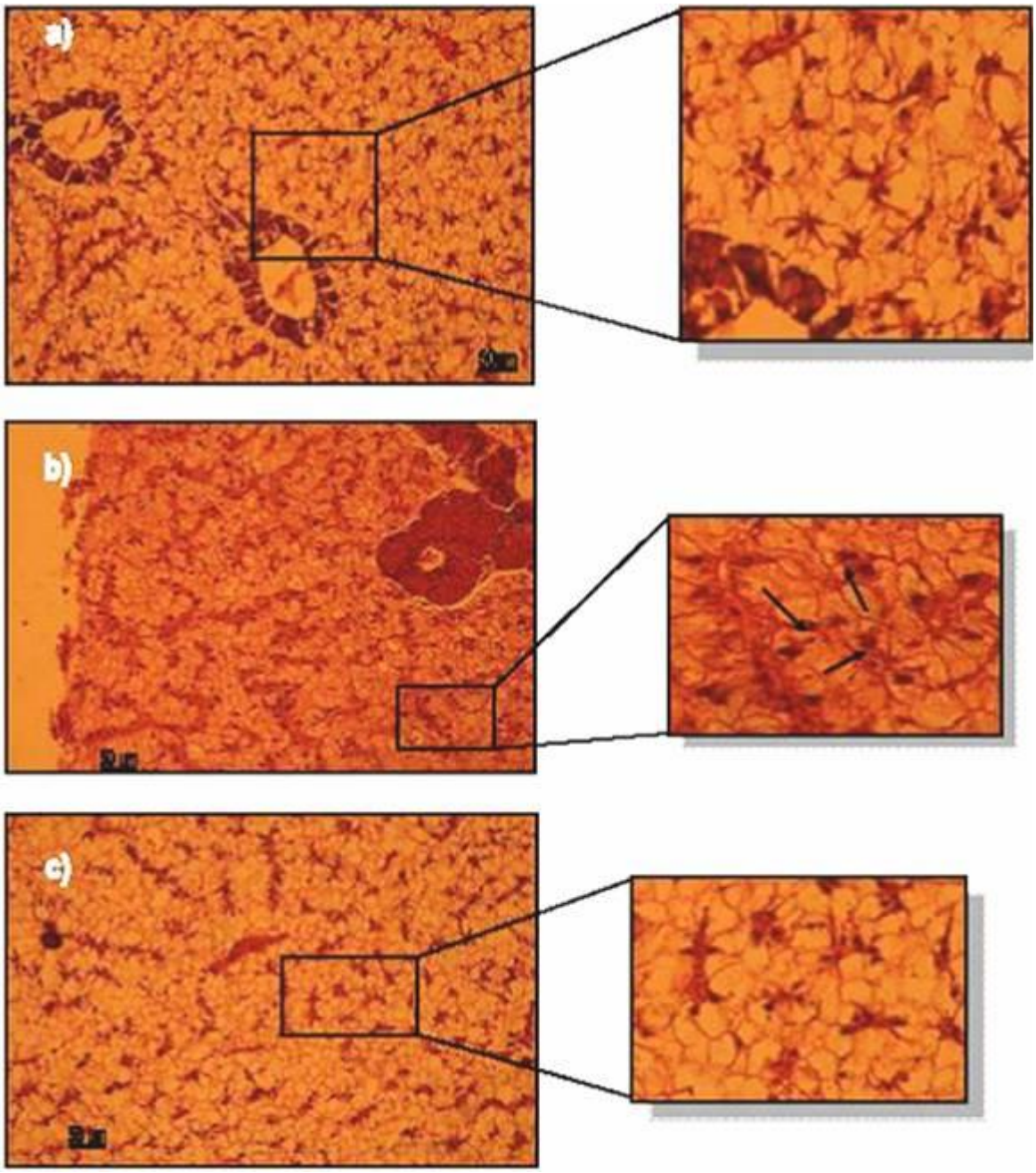

Figure 8. Hepatocytes from fish fed experimental diets. **a)** Diet supplemented with 300 mg/kg (MI) showing obliteration in normal arrangement of hepatocytes, distortion of liver cell orientation and shrinkage of hepatocytes. Bars indicate 50 µm; (**b)** Diet supplemented with 400 mg/kg (MI) showing pycnotic nuclei and cytoplasmic vacuolation, Bars indicate 50 µm; (**c)** Diet supplemented with 500 mg/kg (MI) showing increases in the intensity of pycnotic nuclei and cytoplasmic vacillation with intensive fatty infiltration. Bars indicate 50 µm.

revealed normal consecutive developmental stages of cytoplasmic oocyte to vacoulized and vitellogenic cell for female fed diets supplemented with 300 mg/kg (MI). Atretic oocytes for mature stage (vacoulised and vitellogenic stage) can be detected randomly in which the nucleus was reduced and cytoplasm lost its natural appearance with vacuoles of different size.

In red tilapia, increase of (MI) concentration from 400 to 500 mg/kg diets, revealed many type of atresia with specific feature such as degeneration of the nucleus, liquification of cytoplasm and yolk and also hypertrophy of the follicular cell which acted as phagocytic cells and invaded the oocyte from out side.

Increased number of atretic oocyte, lead to reduction in

plasma steroids levels which included testosterone and 17, 20- dihydroxyprogesteron in both sexes, as well as 11-ketotestosterone in male and testosterone and 17-estradiol in female. MI is a structural component of biological membranes in the phospholipid form, Inositol phospholipds and their metabolites are universal second messengers in the signal transduction pathway.

Biochemical functions of phosphatidylinositol include the mediation of cellular responses to external stimuli, nerve transmission and the regulation of enzyme activity through specific interactions with various proteins (Chang et al., 2001)

The observation of the histological section in the testis of red tilapia showed a marked asynchronous development in spermatogenesis within the same testis; all developmental cell stage was observed inside the lobules. The testis of red tilapia fed diets supplemented with 300 and 400 mg/kg diets were not affected by dietary treatments and the results revealed normal development of active spermatogenesis process at maturing; nearly ripe and spawning stages, while the testis of fish fed diets supplemented with 500 mg/kg diets showed resorption of spermatozoa and thick lobules boundary tissue.

Deformation of spermatozoa as a result of deformation of sertoli cell may lead to interruption of mechanical action of these cells by pushing ripe sperms down to sperm duct which leads to destruction and degeneration of spermatozoa (Billard, 1992; Han-Ping et al., 2008; Esther et al., 2010).

The liver of red tilapia fed diets that contained 300 mg/kg revealed that hepatocytes had distracted membrane and faintly stained cytoplasm, with common pycnotic nuclei. The results revealed obliteration in normal arrangement of hepatocyte, distortion of liver cell orientation and shrinkage of hepatocyte. With increase in the dose of MI to 400 mg/kg, the microscope observation showed pyenotic nuclei and cytoplasmic vacuolation. With increase in the dose of (MI) to 500 mg/kg diets lipid infiltration and lipid metabolic disturbance resulted in the accumulation of lipid in liver, decreased hepatic lipoprotein output and intestinal lipodystrophy; this attributed to interference with chylomicron assembly and secretion and thus the impairment of lymphatic transport of dietary fat (Hegsted et al., 1973; Chu and Geyer, 1988).

Peres et al. (2004) reported that Nile tilapia can probably synthesize inositol in quantity sufficient to meet the requirement for normal growth, feed utilization, immune function and disease resistance but insufficient to prevent alteration of lipid metabolism.

Conclusion

Inositol is widely distributed in common food ingredient. Practical diets should contain sufficient level of this vitamin to meet various metabolic needs. The recommended dose is 400 mg/kg diets to obtain normal males. Also the results explained the mechanism of inositol action through reproduction disfunctions and recruitment failure which occurred at dose more than 400 mg/kg. This may caused a reduction in population size.

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