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In vivo response of melatonin, gonadal activity and biochemical changes during CYP19 inhibited sex reversal in common carp *Cyprinus carpio* (L)

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ABSTRACT

CYP19 aromatase is the key enzyme in vertebrate steroidogenesis, catalyzing the conversion of C19 androgens to 17β-estradiol (E_2). The objective of the present study was to assess the effect of the CYP19 inhibitors (AIs) fadrozole and anastrozole on gonadal development and sex differentiation in *Cyprinus carpio* and investigate the possible involvement of *in vivo* melatonin (MLT) production during sex differentiation. The CYP19 activity in 30 day-post fertilized (30 dpf) fingerlings was inhibited by treating with fadrozole and anastrozole in doses of 100 mg/kg and 200 mg/kg of feed. Gonado-somatic-index (GSI) of fish decreased ($P < 0.005$) and the changes in GSI was dose dependent. Serum testosterone (T) concentration increased ($P < 0.001$) after AI treatments and was negatively correlated with serum E_2 concentration which decreased ($P < 0.005$). Morning serum MLT concentration decreased during the period of inhibited CYP19 activity with a positive correlation with E_2 concentration. Sex-ratio in anastrazole (200 mg/kg) treated fish were 98.1% males while with fadrozole treatment at the same dose resulted in a 97.1% masculinization. Histological examination of fadrozole-treated fish gonads resulted in detection of atretic follicles and intensified spermiation. The protein and lipid production was depressed in AIs-treated fish. The results suggested that fadrozole and anastrozole both effectively inhibited oogenesis and ovarian development in *C. carpio* accelerating testicular formation. There was a physiological correlation between CYP19 activity, E_2 and MLT synthesis during gonadal development and sex differentiation.

1. Introduction

Fish are descendants of ancestral urochordates (Tunicates) or cephalochordates (Amphioxus) and are the largest group of vertebrates comprising about 28,700 fish species (Nelson, 2006; Piferrer and Guiguen, 2008). Modes of reproduction in fish are of an extreme diversity because of the great number of species and also vary as a result of the large variety of aquatic environments inhabited. Reproductive strategies of aquaculture fish species are diversified and require suitable management practice for production enhancement (Devlin and Nagahama, 2002; Jalabert, 2005). Such diversity may relate to sexuality, and specific features of gametogenesis such as the duration of vitellogenesis, and egg morphology, fecundity and multiple spawning (Devlin and Nagahama, 2002; Esther et al., 2010; Rüdiger et al., 2010). Research conducted during the last two decades on reproductive mechanisms in the teleost has certainly increased knowledge concerning fish

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reproduction and its amenability for aquaculture (Jalabert, 2005; Pandian and Sheela, 1995; Singh and Pandey, 1995; Devlin and Nagahama, 2002; Guiguen et al., 2010; Singh, 2012).

Reports on normal gonadal differentiation, and steroid-induced masculinization with androgens, aromatase inhibitors or estrogen receptor antagonists, temperature-induced masculinization in gonochorist fish and hermaphrodite species are available (Devlin and Nagahama, 2002; Guiguen et al., 2010; Singh, 2012). Fadrozole and anastrozole are reversible competitive inhibitors of CYP19 that can affect E_2 biosynthesis in the mammals and fish (Guiguen et al., 2010). Aromatase activity is understood as an important modulator of E_2 production and is important in regulation of reproductive processes (Guiguen et al., 2010). There is increasing evidence that any of a large number of chemicals present in the environment, including some fungicides and isoflavonic phytoestrogens, can also inhibit aromatase activity (Vingaard et al., 2002). Most of the *in vitro* studies that have investigated the steroidogenic ability of the testes, however, have demonstrated testes are incapable of secreting estrogens, and therefore, lack aromatase activity (Ankley et al., 2001; Guiguen et al., 2010). Profiling the physiological effects of fadrozole and anastrozole has occurred in fish (Ankley et al., 2002; Zerulla et al., 2002; Bhandari et al., 2004a; Fenske and Segner, 2004; Navarro-Martin et al., 2009; Guiguen et al., 2010), frogs (Olmstead et al., 2008), and rodents (Huddleston et al., 2006). By inhibiting aromatase activity, fadrozole decreases serum E_2 concentrations (Ankley et al., 2002; Guiguen et al., 2010; Singh, 2012), affecting gonadal development and potentially inducing sex reversal for masculinization in fish (Fenske and Segner, 2004; Singh, 2012). Earlier reports have shown that CYP19 enzyme is responsible for the conversion of progesterone to estrogen (Guiguen et al., 2010; Singh, 2012). Aromatase mRNA studies in fish have revealed that CYP191a and CYP191b are preferentially, but not exclusively, expressed in the gonads and brain, respectively (Piferrer and Blazquez, 2005). The physiological aspects of sex differentiation in fish are still not clearly understood because it is not the only important aspect of CYP19 enzyme activity which regulates sex differentiation or it is also associated with some neuroendocrine mechanism possibly involving pineal activity. In eels, MLT implants decrease LHβ and FSHβ subunit synthesis as well as plasma concentrations of some sexual steroids (Sebert et al., 2008). In the cultured carp hypothalamus, MLT reduces dopamine concentrations which result in an increase in LHβ subunit synthesis (Popek et al., 2006). Recently, a significant role of MLT was reported in regulation of annual testicular events in *Catla catla* (Chattoraj et al., 2009). Melatonin exerts pro-gonadal or anti-gonadal actions during reproductive development and maturity in fish (Singh, 2009; Falcón et al., 2010; Singh et al., 2012), however, its role in sex differentiation is not yet known.

Common carp, a widely cultured fish in most parts of the world, is an important food fish, however, precocious maturity and the prolific breeding nature of this fish limits its aquaculture potential. Recognition of physiological and endocrine processes responsible for gonadal development and sex differentiation is important in controlling maturity and breeding in this fish species. Utilizing the knowledge about the biological action of the aromatase inhibitors, fadrozole and anastrozole, a simple method to control maturity and prolific breeding of common carp that typically results in stunted growth and impaired production was evaluated in the present study. There was also assessment of whether endogenous MLT was involved in the process of gonadal development and differentiation.

2. Materials and methods

The fish were acclimatized in glass aquaria (150 L capacity) duly fitted with high quality aerator filters and thermostat heaters so as to maintain sufficient oxygenated condition and temperature of $26 \pm 1\,^{\circ}C$. Fingerlings ($n = 300$; 30 dpf) of common carp (mean weight 2.45 ± 0.64 g) obtained from the hatchery of National Bureau of Fish Genetic Resources, Lucknow were treated with potassium permanganate (5 ppm) to minimize the bacterial infection. After 7 days of acclimation to the laboratory conditions, the fingerlings were equally divided into five experimental groups in triplicate. For mixing CYP19 inhibitors (AI), fadrozole and anastrozole (SIGMA Life Sciences), were dissolved in a minimal volume of 95% absolute alcohol and the required volume of the AI solutions was sprayed over feed (Tyio Pvt. Ltd.) to provide the required doses of 100 mg/kg and 200 mg/kg of feed which was then dried in an oven at 45 °C. This AI-incorporated feed containing different doses of fadrozole (F_1 and F_2) and anastrozole (A_1 and A_2) was provided to the fingerlings daily up to satiation twice a day for 90 days. The control group of fingerlings (C) was provided food that contained no fadrozole and anastrozole. After 90 days, all fish were measured for length and weight (F_L and F_W) with the use of a digital caliper meter scale and a digital weighing machine (Denver Instrument, Germany). Specific growth rate ($SGR\%^{-day}$) and condition factor (K) were calculated with the formula: $SGR\,(\%^{-day}) = 100 \times ({}^{log}\text{final weight} - {}^{log}\text{initial weight}/n$ (number of days of treatment); condition factor (K) = (weight in g)/(total length in cm)$^3 \times 100$. The GSI was determined as: GSI = gonad weight/body weight × 100. The GSI was calculated with a precision of 0.01 g to estimate any changes in fish maturity.

Fish ($n = 20$) of each experimental groups (F_1, F_2, A_1, A_2 and C) were anesthetized by immersion in water containing 100 mg tricaine methanesulfonate (MS-222) per liter (Merck, Germany), buffered with sodium bicarbonate (100 mg/L). Blood of the anaesthetized fish was drawn at 8.30 A.M. from the caudal vein which was then pooled for each experimental group. About 2 ml blood was separated and placed in non-heparinized tubes and left to clot at 4 °C for 15 min. Afterwards, tubes were centrifuged at 3000 rpm using an Eppendorff Centrifuge for 10 min to obtain serum. The serum was separated into aliquot which was stored at −80 °C until used for hormone, protein and lipid analyses. The remaining 1 ml of blood was kept in blood collection tubes containing 0.05 ml of 15% EDTA for a hemoglobin test and stored at −4 °C until assayed.

Fish of the different experimental groups were dissected after collecting blood and gonads were removed

immediately afterwards and fixed in 10% natural buffer formalin solution, dehydrated in different grades (30%, 70%, 90% and absolute) of ethanol and embedded in paraffin wax. Sections (5–7 μm thick) were cut, dewaxed, and dehydrated with different grades of ethanol, stained with Hematoxylin and Eosin, dehydrated and mounted in Canada balsam (Merck, Darmstadt, Germany). Sections were examined under a compound microscope (Olympus Co., Japan).

The serum protein content of fadrozole- and anastrozole-treated and control fish was determined by the Lowry method (Lowry et al., 1951) using a kit (Bangalore GENEI/Qualigens). Bovine serum albumin (BSA) was used in different concentrations for preparing the standard curve and extrapolating experimental values. An absorbance reading was taken with a spectrophotometer (Tecan, USA) at an optical density of 660 nm. The gonadal protein was determined similarly using 2 mg of gonadal tissue. For the measurement of serum lipid, blood was collected into tubes containing 1 mg EDTA/ml. Serum cholesterol and triglycerides concentrations were analyzed using the method of Stein (1986). At a similar time, gonads collected from the control as well as experimental fish were evaluated for total lipid content using the method of Bligh and Dyer (1959). Observations for lipid values were taken on a spectrophotometer at 440 nm wavelength. E_2, T and MLT concentrations of treated and control fish were estimated by enzyme-linked immuno-absorbent assay (ELISA). Serum E_2 and T were assayed by using kits (Enzo Life Science, India) as per the detailed protocols provided by the manufacturer. The cross reactivity of the assay and purity was 100%. The sensitivity for T assay was 5.67 pg/ml. The sensitivity or limit of detection of the E_2 was 14.0 pg/ml. Serum MLT was estimated using the ELISA method (MP Biomedical, India) and the readings were taken at an optical density (OD) of 405 nm using an ELISA reader. The assay procedure for MLT followed the basic principle of competitive ELISA where competition between a biotinylated and non-biotinylated antigen for a fixed number of antibody binding sites was utilized in the process. The amount of biotinylated antigen bound to the antibody was inversely proportional to the analyte concentration of the sample. The bound biotinylated antigen was determined by the use of anti-biotin alkaline phosphatase as marker and p-nitrophenyl as substrate. Quantification of unknown samples was achieved by comparing the enzymatic activity of unknowns with a response curve prepared by using known standards. The sensitivity of MLT assay was 1 pg/ml. The MLT assay was highly sensitive and had excellent specificity for the detection of MLT. No significant cross-reactivity or interference between MLT and analogs was detected.

All data were calculated and presented as means ± standard deviation. Student's *t*-test was calculated for comparisons between control and treated groups. The coefficient of variation was also calculated so as to ascertain the efficacy of the treatments. Further, 'Chi square' (χ^2) tests were performed for calculating the variation in sex ratio in the treatment groups from the expected Mendelian sex ratio. Data were analyzed by one way analysis of variance (ANOVA) using a computer programmed software, statistical package for social science (SPSS).

3. Results

In general the mortality ranged from 7.5% to 18.5% in different groups during the experimental period but was not different for any particular treatment group. The large dose of fadrozole and anastrozole decreased growth rate. The SGR% was reduced ($P < 0.005$) to 0.76 ± 0.09 from the control value of 1.19 ± 0.07 (Fig. 1). The coefficient of variation for the anastrazole-treated group was 7.63 and was less than the coefficient of variation of 7.73 in the fadrozole-treated group. Condition factor of anastrazole-treated fish was less (1.98 ± 0.91) in fish treated with the larger dose of anastrozole while in case of the larger dose of fadrozole it was 2.71 ± 0.67. However, the condition factor in fadrozole-treated fish was greater than that for the anastrazole-treated fish (Fig. 1). The coefficient of variation for fadrozole treatment was 3.94 which was greater than the value of 3.81 in the anastrazole-treated group. The gonado-somatic index (GSI) was less ($P < 0.005$) after anastrazole treatment with the dose of 200 mg/kg of feed. With the smaller dose of fadrozole (F_1), GSI was 3.57 ± 0.78 while with the larger dose (F_2), it was 3.18 ± 0.67 at 90 days (Fig. 2). The GSI value after anastrozole treatment was suppressed to a greater extent as compared to the fadrozole-treated group. The coefficient of variation for GSI was 19.58 in the anstrazole-treated group, while it was 21.48 in the fadrozole-treated group indicating that the effect of fadrozole on GSI had more commercial potential (Fig. 2). The sex ratio after fadrozole and anastrazole (200 mg/kg of feed) treatments revealed that there was 97.1% and 98.1% masculinization, respectively. There was 1% intersex common carp with the smaller dose of fadrozole treatment (F_1) and 1.5% intersex after the smaller dose of anastrozole treatment (A_1) (Fig. 3). The coefficient of variation for males was 10.03 and 5.88 for females after anastrozole treatment which was less than the fadrozole treatment where the coefficient of variation was 19.98 for males and 9.59 for females. This indicated that masculinization with the fadrozole treatment was more consistent. The χ^2 value for sex ratio was 22.67 which was almost equal to degrees of freedom (df) 22.46 indicating the deviation in sex ratio from the expected Mendelian sex ratio was highly significant (Fig. 3).

Changes in the serum protein concentration was 19.04 ± 1.22 mg/ml with the larger dose of fadrozole (F_2) and the value was highly significant ($P < 0.001$). With the large dose of anastrozole (A_2), the serum protein concentration was 17.45 ± 1.09 mg/ml which was less ($P < 0.005$) than the control value of 27.6 ± 1.10 mg/ml. Gonadal protein concentration was less after AI treatments and was 14.74 ± 1.12 mg/g with the larger dose of fadrozole and 13.46 ± 1.03 mg/g with the larger dose of anastrozole, respectively. However, there was only a difference in gonadal protein concentration with the larger dose of anastrazole (Fig. 4). The serum cholesterol concentration was less (172.0 ± 3.47 mg/dl) with the larger dose of fadrozole (F_2). With the larger dose of anastrozole, (A_2), serum cholesterol concentration was

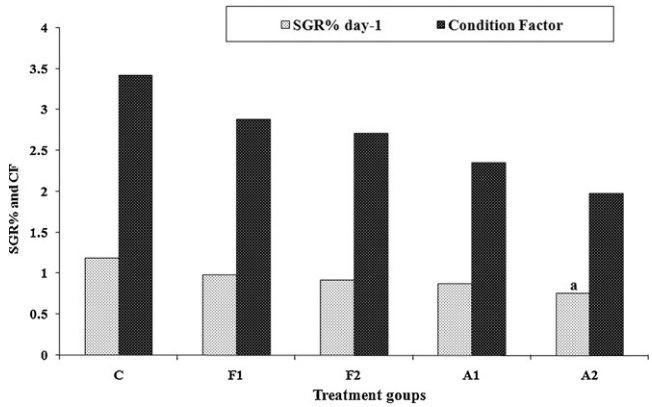

Fig. 1. Specific growth rate (SGR%) and condition factor (CF) after fadrozole and latrozole treatments of *Cyprinus carpio* (significance level: a = $P < 0.005$).

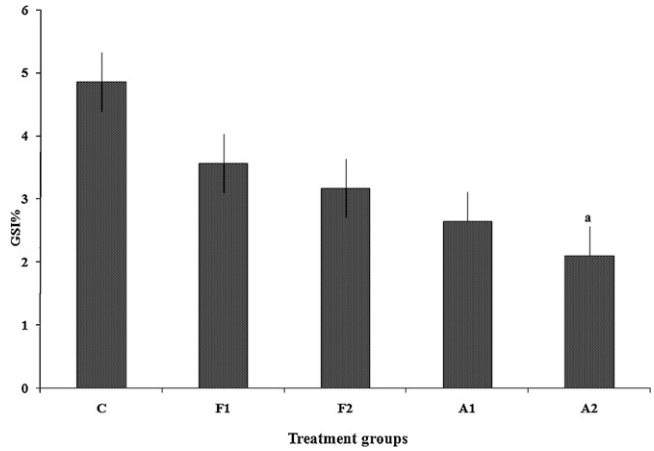

Fig. 2. Effect of fadrozole and anastrazole on gonado-somatic index of *Cyprinus carpio* (significance level: $P < 0.005$).

164.0 ± 3.15 mg/dl and was less ($P < 0.005$) compared to the control value (286.0 ± 4.50 mg/dl). Serum total triglyceride was also less ($P < 0.005$) with the larger dose of fadrozole (F_2) and anastrozole (A_2) as compared to the control value of 254.0 ± 17.08 mg/dl (Fig. 5). Total gonadal lipid was 0.55 ± 0.25 μg/g with the large dose of fadrozole (F_2) while with the larger dose of anastrozole (A_2), gonadal lipid was less ($P < 0.001$) as compared to the control (Fig. 5).

The estimated serum E_2 concentration was less ($P < 0.005$) in fadrozole- as well as anastrozole-treated fish as compared to the control value of 87 ± 4.21 pg/ml (Fig. 6). With the larger dose of fadrozole (F_2), the value was 39 ± 2.13 pg/ml and with the smaller dose of fadrozole (F_1) was 51 ± 3.76 pg/ml. With the larger dose of anastrozole (A_2), serum E_2 was 33 ± 2.48 pg/ml and with the smaller dose (A_1) was 47 ± 3.67 pg/ml (Fig. 6). Serum T concentration was 1030 ± 7.34 pg/ml with the smaller

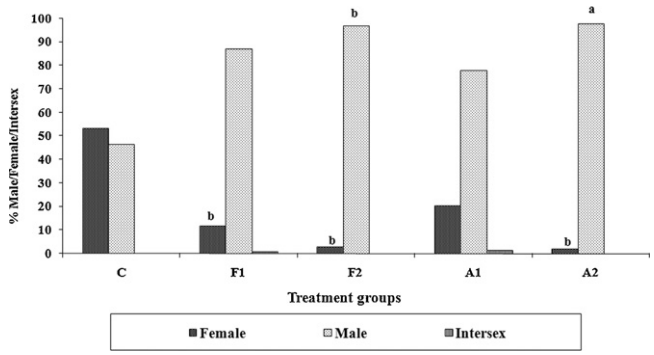

Fig. 3. Sex ratio after fadrozole and anastrazole treatments of *Cyprinus carpio* (significance level: a = $P < 0.005$; b = $P < 0.001$; $\chi^2 = 22.67$).

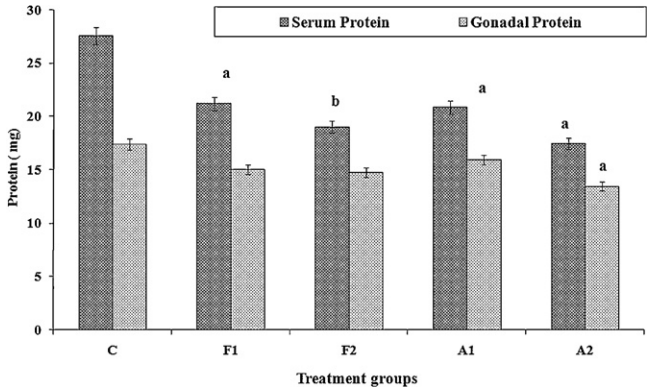

Fig. 4. Serum and gonadal protein after fadrozole and anastrazole treatments of *Cyprinus carpio* (significance level: a = $P < 0.005$; b = $P < 0.001$).

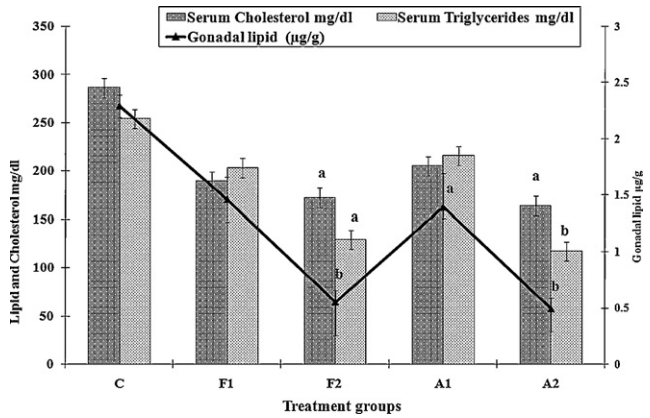

Fig. 5. Serum/gonadal lipid and cholesterol after fadrozole and anastrazole treatments of *Cyprinus carpio* (significance level: a = $P < 0.005$; b = $P < 0.001$).

dose of fadrozole (F_1), and 1286 ± 8.44 pg/ml with the larger dose (F_2). In anastrozole treated fish, the T concentration was 1072 ± 7.23 pg/ml with the smaller dose (A_1) and 1324 ± 8.39 pg/ml with the larger dose (A_2) and was greater ($P < 0.001$) as compared to control values of 892 ± 5.84 pg/ml (Fig. 7). MLT concentration was less ($P < 0.001$) after fadrozole as well as anastrozole treatment. Morning concentration of serum MLT was 20 ± 2.46 pg/ml with the smaller dose of fadrozole (F_1) and 18 ± 1.98 pg/ml with the larger dose of fadrozole (F_2). In anastrozole treated fish, MLT concentration was less with the value of 19 ± 2.15 pg/ml with the smaller dose (A_1) and with the larger dose (A_2) MLT was 14 ± 1.74 pg/ml which was less ($P < 0.001$) than the control value (Fig. 8). The coefficient of

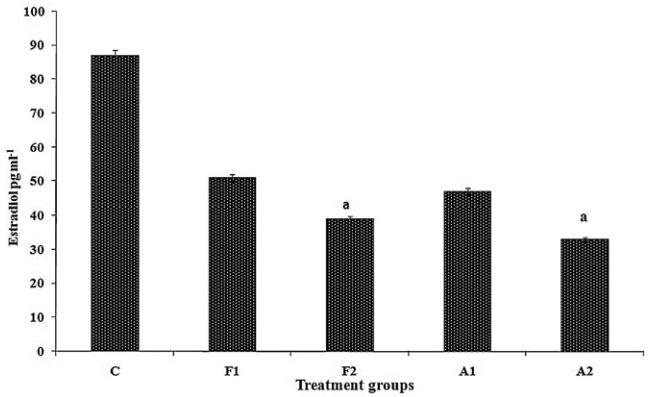

Fig. 6. Serum E_2 after fadrozole and anastrazole treatments to *Cyprinus carpio* (significance level: a = $P < 0.005$).

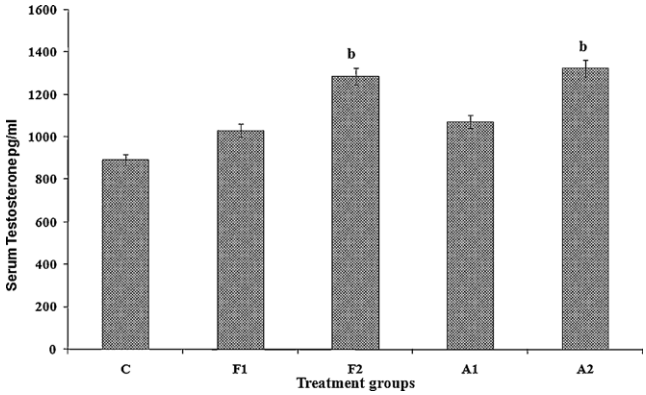

Fig. 7. Testosterone concentrations after fadrozole and anstrazole treatments of *Cyprinus carpio* (significance level: b = $P < 0.001$).

variation indicated serum MLT was more effectively suppressed with anastrozole treatment (A_2) as compared with fadrozole (Fig. 8).

Structure of gonads showed many changes in the AI-treated fish as compared to the control fish. Male fish possessed elongated paired testes. The ovaries of fadrozole-treated fish were identified as Stages II and III and were less mature than those from the control group, which were in Stage IV. Histological examination of ovaries of control females showed ovarian structures filled with normal synchronously developing oocytes in the late perinuclear stage (Fig. 9A). Conversely, fadrozole-treated females had gonads with some atretic oocytes, which were in the process of absorption by phagocytes. An increase in the number of pre-ovulatory atretic follicles was observed in females treated with the larger dose of fadrozole. There were no discernable alterations in external morphology, including secondary sexual characteristics, of fish treated with fadrozole or anastrazole. Histological examination of testes of control fish revealed III and IV development stages (Fig. 9B). The smaller dose of fadrozole and anastrazole resulted in intersex individuals with the presence of vitellogenic follicles, spermatocytes, and oocytes in the same gonad (Fig. 9C). The anastrazole treatment resulted in functional males having testes, which were indistinguishable in structure from those of normal males, but larger in size, and all stages of spermatogenesis had been completed including accumulation of large amount of sperm in the seminiferous tubules (Fig. 9D).

4. Discussion

Results of the present study delineated that specific growth rate of *C. carpio* was reduced after fadrozole and anastrozole treatments. However, there was little variation in condition factor and it was above the value of 1 in all the treatment groups. However, no significant difference in body weight and length of bluegill sunfish, *Lepomis macrochirus*, has been report after the AI treatments (Gao et al., 2009). Further, findings in the present study indicate there was a depressed GSI after fadrozole and anastrazole treatments of common carp. There was little variation in GSI between the control and AI-treated Japanese medaka, *Oryzias latipes* (Sun et al., 2007). Ankley et al. (2002) have also reported that there is no significant effect of fadrozole on GSI of the fathead minnow, *Pimephales promelas*. However, Li et al. (2006) reported that fadrozole treatment decreases the GSI value in the red-spotted grouper, *Epinephelus akaara*.

Fadrozole and anastrozole were found to decrease, lipid, cholesterol and triglyceride concentrations in the present study. However, there was very little change in the serum

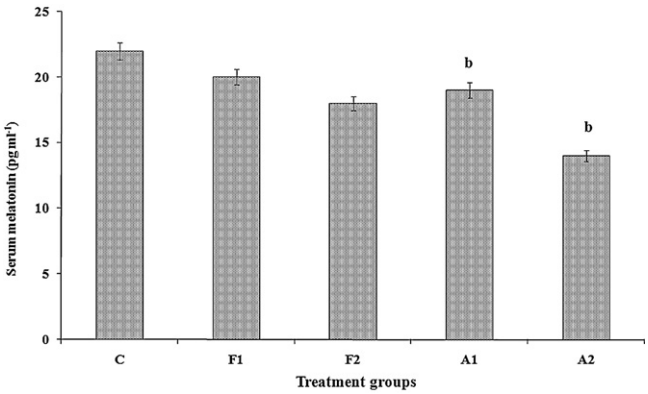

Fig. 8. Serum MLT concentrations after fadrozole and anastrazole treatments of *Cyrpinus carpio* (significance level: b = $P < 0.001$).

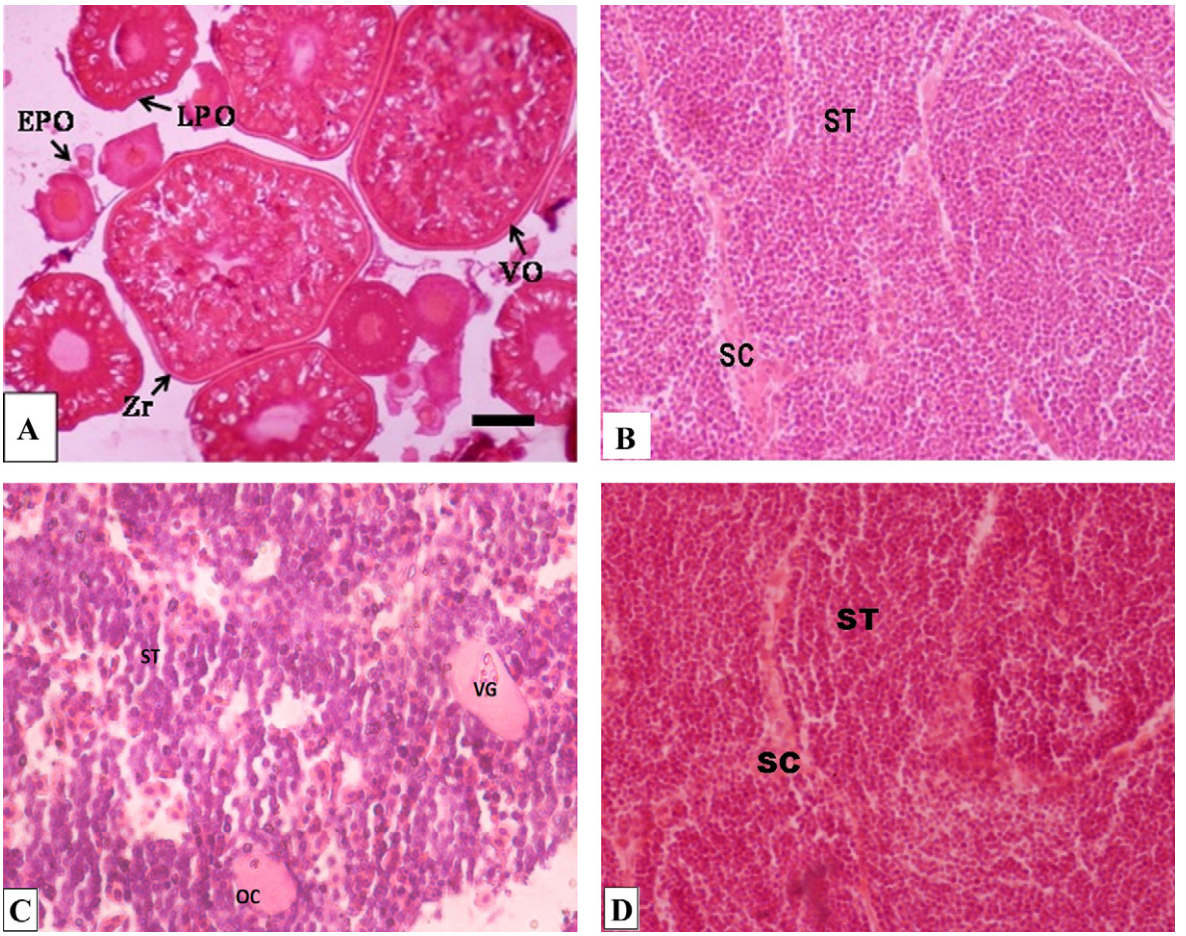

Fig. 9. (A) Histological section of control ovary showing early perinuclear oocytes (EPO), late perinuclear oocytes (LPO), vitellogenic oocytes (VO), zona radiata (Zr), scale = 20 μm; stain – H&E. (B) Histological structure of control testes showing spermatid (ST), spermatocytes (SC) and lumen (L), scale = 20 μm, stain – H&E. (C) Histological structure of fadrozole treated intersex gonad (testes) showing oocyte (OC) spermatid (ST), vitellogenic follicle (VG), scale = 20 μm, stain – H&E. (D) Histological structure of anastrazole treated gonad (testes) showing active spermatocytes (SC) and spermatid (ST), scale = 20 μm, stain – H&E.

and gonadal protein concentrations. In mammals (rats), anastrozole affects the metabolic process of protein and lipid oxidation (Mauras et al., 2000). In fish, lipid and protein concentrations influence gonadal development and maturation (Rainie and MacLatchy, 2007). Nevertheless, there is little information about lipid and protein changes due to the action of CYP19 inhibitors or MLT. Reduced concentrations of lipid, cholesterol and triglycerides in the present study indicate fadrozole and anastrazole inhibited lipid and cholesterol production which was specific to the males during gonadal development and sex differentiation for masculinization (Rainie and MacLatchy, 2007; Singh et al., 2012).

Common carp treated with fadrozole and anastrazole had increased plasma concentrations of T in the present study. Treatment inhibited CYP19 increased T concentrations in males by blocking conversion of T to E_2 (Jensen et al., 2001; Guiguen et al., 2010; Singh, 2012). An additional observation of interest in the process of masculinization was the occurrence of a relatively unique histopathology in the gonads. Specifically, there was a notable, T concentration-dependent enlargement of the seminiferous tubules accompanied by an abundant accumulation of sperm in the lumina. The marked accumulation of sperm in testes of fadrozole-treated fish is positively related to enhanced sperm production with increased plasma T (Afonso et al., 2000; Guiguen et al., 2010).

Afonso et al. (1999a) found that fadrozole (AI) injection at dosages of 0.1, 1.0, and 10.0 mg AI/kg body weight decreased E_2 concentrations. In the present study, serum E_2 concentration was significantly reduced after fadrozole and anastrazole treatment. Mikolajczyk et al. (2007), however, found that there is no significant difference in concentrations of E_2 in the blood plasma and hypothalami after fadrozole treatment of gold fish and common carp. Li et al. (2005) found letrozole (5 mg/kg body weight) decreased serum concentrations of E_2 which was associated with sex inversions induced by aromatase inhibitor. Bhandari et al. (2004b) reported that treatment with fadrozole decreased plasma E_2 in the honeycomb grouper, *Epinephelus merra* which further corroborates findings in the present study. Li et al. (2006) also reported that

fadrozole decreases serum E_2 concentrations in the red-spotted grouper, *Epinephelus akaara*. Anastrozole in doses of 100 mg/kg and 200 mg/kg suppressed E_2 and promoted T biosynthesis in the present study which is supported by earlier reports (Mauras et al., 2000; Hilleson-Gayne and Clapper, 2005; Sherri-Ann et al., 2009; Guiguen et al., 2010; Singh, 2012). The effects of fadrozole on plasma steroid concentrations in male and female common carp and other fish such as coho salmon and fathead minnows have been previously reported (Mauras et al., 2000; Hilleson-Gayne and Clapper, 2005; Sherri-Ann et al., 2009; Guiguen et al., 2010). There are several reports which indicate that the use of aromatase inhibitors induces sex reversal in fish (Afonso et al., 1999a,b, 2000; Sun et al., 2007; Guiguen et al., 2010; Singh, 2012).

The results of the present study suggest that inhibition of aromatase delayed the expected maturation of the gonads over the duration of the fadrozole and anastrazole treatments. The histological assessment of ovaries of fadrozole-treated fish has been reported to decrease maturation of oocytes and increase the number of preovulatory artretic follicles (Ankley et al., 2002). But in some fish such as bluegill sunfish and tilapia, AI did not influence ovarian or testicular structures between control and AI-treated fish (Afonso et al., 2001; Gao et al., 2009). Furthermore, in the honeycomb grouper, AI inhibits production of E_2 *in vivo*, causing oocyte degeneration and subsequently the sex inversion from female to male (Bhandari et al., 2004b). Both fadrozole and anastrazole induced functional males with male testicular structure similar to that in normal male in previous studies (Li et al., 2005; Park et al., 2004). AI enhanced masculinization in the present study with advancement in timing at which spermiation began and increased plasma T concentrations. Long-term oral administration of AI results in the onset of spermiation at least 3 months later and with a much larger volume of sperm as compared with the control group (Lee et al., 2001). Masculinization of gonads by aromatase inhibitors occurred in several gonochoristic fish including Chinook salmon, *Oncorhynchus tshawytscha* (Piferrer et al., 1994), rainbow trout, *O. mykiss* (Guiguen et al., 1993), tilapia, *Oreochromis niloticus* (Guiguen et al., 1993; Kwon et al., 2000), and Japanese flounder, *Paralichthys olivaceus* (Kitano et al., 2000). From a practical perspective, findings in the present study indicate fadrozole and anastrozole retarded gonadal development which may be important particularly when unwanted gonadal development and reproduction needs to be prevented. In common carp, morphological and physiological changes during sexual maturation, sexual dimorphism and growth cessation are important determinants to product quality. Therefore, the development of technique which could prevent or retard these changes, under culture conditions, is desirable.

Reports are available suggesting antigonadal activity of MLT in fish reproduction (Chattoraj et al., 2009; Tamura et al., 2009; Singh et al., 2012). However, information about the role of MLT in sex differentiation is still not known. Serum MLT concentrations in the present study were decreased in anastrazole-treated common carp. Changes in concentrations of MLT due to the action of anastrazole and fadrozole are a topic of discussion. Nevertheless, findings in the present study demonstrate a close correlation of MLT and E_2 concentrations and aromatase activity which suggests that MLT is directly involved in sex differentiation. Result of the present study supported the earlier work where involvement of MLT was implicated in sex changes in the ricefield eel, *Monopterus albus* (Shi, 2005).

5. Conclusion

The results of the present study demonstrated that aromatase inhibitors, fadrozole and anastrazole, inhibited early oocyte development in female common carp and it was concluded that aromatase played a pivotal role in inhibiting oogenesis during the early stage of ovarian development. Fadrozole and anastrazole both were found to have potent masculinizing effects. During gonadal development and sex differentiation, the CYP19 inhibitors brought about marked increases in the T concentrations leading to testicular development and spermiation. Furthermore, the activity of aromatase was also found to directly modulate MLT production delineating that MLT has important role in gonadal sex differentiation.

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