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Research Article

The effect of Letrozole and Letrozole-Chitosan nanoparticles on masculinization of Rainbow trout larvae (*Oncorhynchus mykiss*)

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Abstract

This study aimed to investigate the efficiency of conjugating chitosan nanoparticles to letrozole (LET) on masculinization of rainbow trout larvae, *Oncorhynchus mykiss*. For this purpose, two treatments of LET, 1.5 and 3 mg/kg food, and two treatments of letrozole-chitosan nanoparticles (LET-CS), 0.5 and 1.5 mg/kg were performed with a control group. Treatments began immediately after active larval swimming and continued for 60 days. The results showed that the amount of testosterone during the trial increased significantly ($p<0.05$), so that the highest amount of testosterone was observed in 3 mg/kg LET and 1.5 mg/kg LET-CS. However, estradiol was untraceable in all treatments. About 66.6, 73.33, 73.33, 76.66 and 43.33% males were observed in 1.5 mg/kg LET, 3 mg/kg LET, 0.5 mg/kg LET-CS, 1.5 mg/kg LET-CS and control, respectively. Despite the drug dosage in LET-CS treatments was half that of LET treatments, the sex ratio did not change among treatments ($p\geq 0.05$), which showed a positive effect of CS in effectiveness of LET. In general, it can be stated that using CS for the administration of LET could be effective in inhibiting the production of estradiol, increasing the amount of testosterone, and thus changing the sex ratio of rainbow trout.

Keywords: Aromatase inhibitor, Letrozole, Chitosan nanoparticles, Rainbow trout, Sex reversal

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Introduction

Sex reversal of fish has become more in demand in recent years due to several benefits, such as growth improvement and elevation of feed efficiency. In many species, females grow faster than males and maturity may occur in males earlier than they reach to marketable size (Piferrer, 2001). In rainbow trout, *Oncorhynchus mykiss***,** breeding, all-female culture is considered to increase production efficiency and prevent the decrease of fish quality due to early maturation in males (Bye and Lincoln, 1986). Direct feminization is made by 17β-estradiol in different methods, including diet and immersion (Piferrer, 2001; Weber *et al*., 2019). However, in many countries, because of community concerns about consumption of directly manipulated fish, the application of hormones for the production of monosex population of rainbow trout or other fish is prohibited. Therefore, in the breeding of this fish, first, a XX male (neomale), is produced through the masculinization method, and then the all-female population is produced by crossing neomales with natural XX females (indirect feminization). Although the indirect method is costlier and time-consuming, the biggest advantage of it is that market fish have never been treated with steroids directly. In addition, monosex farming can achieve significant economic benefits in the long run and is a promising way to increase production efficacy (Budd *et al*., 2015). The synthetic androgenic hormone 17 α-methyltestosterone (MT) has been used to masculinize many fish species, including rainbow trout (Pandian and Sheela, 1995; Budd *et al*., 2015) and the protocol for masculinization of rainbow trout (3 mg/kg of hormone for 60 days since active feeding) is well defined (Weber *et al*., 2019). On the other hand, most sex-reversed trout with hormonal treatment show a kind of delectable sperm duct (Tsumura *et al*., 1991), in which the fish must be killed and the gonad removed from the body to extract the sperm. This causes problems in managing fish reproduction. Studies have shown that reducing the dose of the hormone to 0.5-1 mg/kg reduces sperm duct deformity by up to 20% without significantly changing the percentage of masculinization (Cousin-Gerber *et al*., 1989; Arslan *et al*., 2012).

Aromatase plays a major role in the final stages of estrogen production and catalyzes the conversion of androstenedione and testosterone to estrone and estradiol, respectively (Haynes *et al*., 2003). Thus, aromatase activity determines the ratio of estrogen to estradiol in gonads and thus determines their sexual phenotype through the expression of the *cyp*19a gene (Nakamura *et al*., 2003; Blázquez *et al*., 2008). Aromatase inhibitors (AI) are used in humans to treat breast cancer in postmenopausal women (Howell et al., 2005; Bhatnagar, 2007). These inhibitors bind to the binding site of the aromatase enzyme and prevent its activity. Inhibition of aromatase synthesis by an aromatase inhibitor in fish can cause fish masculinization (Piferrer *et al*., 1994). Today, in a large number of bony fishes (Doering *et al*.,

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2019) aromatase inhibitors including letrozole (LET) (Qin *et al*., 2020), tamoxifen (Singh *et al*., 2015), and fadrozole (Babiak *et al*., 2012; Evliyaoğlu *et al*., 2019) have been tested. LET is one of the most common non-steroidal aromatase inhibitors for the treatment of breast cancer (Lamb and Adkins, 1998). This drug has been used for sex reversal in tilapia (Afonso *et al*., 2001; Betancur López *et al*., 2014), rainbow trout (Xu *et al*., 2021), fighting fish (Katare *et al*., 2015), common carp (Singh and Srivastava, 2015), Ussuri catfish (Wang *et al*., 2019), Yellow catfish (Shen *et al*., 2015), protogynous grouper (Ranjan *et al*., 2013), and blue drum and gynogenetic yellow drum (Qin *et al*., 2020). The use of LET alone (Xu *et al*., 2021) or in combination with MT (Ranjan *et al*., 2013) can have good results in fish masculinization.

Due to its hydrophobicity and low bioavailability of LET in the gut (Azandaryani *et al*., 2019), binding it to various carriers including nanoparticles can increase the efficiency and the effectiveness of the drug (Mondal *et al*., 2008; Gomathi *et al*., 2017). Chitosan is one of the most abundant natural polymers and is a base compound with a positive surface charge (Gomathi *et al*., 2017). This property allows it to be easily combined with fats, cholesterol, proteins, metal ions, and negatively charged surface molecules (Chatelet *et al*., 2001). Due to its biodegradability and compatibility properties, chitosan is very promising for drug delivery such as LET and can reduce drug use by increasing its bioavailability (Wisdom *et al*., 2018).

Therefore, the present study was conducted to investigate the possibility of reducing the dose of the LET in masculinization of rainbow trout. This is the first report on the use of LET-CS in this species.

Materials and methods

This research was conducted in the Shahid Motahari Genetics and Breeding Research Center of Cold Water Fish, Yasouj, Iran. The water flow of this center varies between 150-1000 liters per second in different seasons. The water temperature during the study period was between 10 and 12°C and saturated dissolved oxygen was measured 7-9 mg/L.

Preparation of Chitosan and nanochitosan

Preparation of chitosan and nanochitosan was performed in the Khatamalanbia University of technology, Behbahan, Iran. Shrimp shell was used to extract chitosan. For this purpose, shrimp shells were prepared from the fresh shrimp in local market of Hendijan fishing port, and dried after washing and separating the rest of the gut and rinsing with distilled water. Dried shrimp shells were ground and chitosan was obtained during three stages of deionization, protein removal, and deacetylation as described by (Younes *et al.*, 2012). To preparing nanochitosan, Ionic gelation method (Calvo *et al*., 1997; Gomathi *et al*., 2017) was used to prepare chitosan

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nanoparticles. For this purpose, the methods developed by Wisdom *et al*. (2018) has been used with slight modification. In original method, propylene glycol has been used as a solvent, but we have used ethanol instead. In summary, 0.2 g chitosan added to 1 mL acetic acid (Merck) and mixed for 10 minutes and kept at room temperature. Twenty (20) mL of deionized water was added and stirred for 40 minutes with a magnetic stirrer (Alfa, Iran) at 5000 rpm. After that 15 mL TPP triphosphate (Sigma-Aldrich) solution (0.01%) was added to chitosan solution dropwise and pH was adjusted to 5.5 by using 1 N NaOH. Finally, volume was set to 100 mL with deionized water. During the chemical reaction process, the chitosan undergoes ionic gelation and precipitates as spherical particles.

Conjugation of the LET to nanochitosan

100 mg of LET (Aburaihan Pharmaceutical Co.) was added to 100 mL of ethanol 96% and completely dissolved. The drug solution was then added to 100 mL of nanochitosan solution. Nanochitosan solution was added dropwise for at least 30 minutes (Wisdom *et al*., 2018).

Experimental treatments

The usual dosages for masculinization of rainbow trout by MT hormone, i.e. 3 and 1.5 mg/kg food, were selected for LET as an aromatase inhibitor (Weber *et al*., 2019) (Treatments LET), and considering that it was predicted that the required dose would be lower by binding LET to chitosan nanoparticles (Rather *et al*., 2013), the dose of the LET-CS was selected as half of the above values, i.e. 1.5 and 0.5 mg/kg food (Treatments LET-CS). Therefore, 5 groups were designed in this experiment, including Control (ethanol 96% was sprayed on food and used after drying), 2-1.5 mg LET/kg food, 3-3 mg LET/kg food, 4-1.5 mg LET-CS/kg food, and 5-0.5 mg LET-CS/kg food. Each treatment was performed in 3 replicates. Feeding with food containing the drug continued for 60 days after the start of active feeding.

Food preparing

In this experiment, the commercial food of Kimiagran Nutrition Company (Shahrekord, Chaharmahal and Bakhtiari Province, Iran) was used. To prepare the food containing LET, 100 mg of LET was added to 100 mL of ethanol 96% and then the required amount of alcoholic solution of LET was sprayed on the food 3 times. After 48 hours and complete evaporation of alcohol at room temperature, the food was stored in the refrigerator at 4°C. For LET-CS treatments, food was prepared in the same way, with LET-CS solution instead of LET alcoholic solution. Ethanol 96% was sprayed on food for the control treatment (Wisdom *et al*., 2018).

Masculinization process

This study was performed in a completely Randomized Block Design (RBD). Larvae were obtained from normal Iranian rainbow trout broodstock. These brooders selected from healthy 3-4 years old fish. The

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mean initial weight of larvae was 0.133±0.007 g. The larvae were divided into 15 fiberglass troughs (220*46*18 cm) immediately after the start of active swimming and feeding. The water flow rate in the each trough was set to 0.5 liter per second. Each treatment contained three troughs as three replication. One basket was placed in each trough and 1000 larvae were introduced to each basket. After 40 days, they were transferred to 100 L tanks containing 50 L of water. Food containing the drug was given to the treatments for 60 days (d) and then feeding with food without the drug was continued until day 125. Biometric method was used every 10 days during the study period. The feeding rate was based on the amount recommended by the food company and 10 times a day. On the third day of the project, due to heavy rainfall, the incoming water became muddy for about 4 d, and feeding the larvae was stopped. The number of mortality during the project period was recorded daily and was reported at the end of the drug period (60 d) and the end of the maintenance period (125 d) (Betancur López *et al*., 2014).

Hormone measurement

To measure testosterone and estradiol sampling was done twice, on the 30th and 60th day after the drug therapy. For this purpose, in each sampling time, 18 individuals (whole fish) from each treatment (6 samples from each replication) were placed in sampling containers on ice and as soon as possible were transferred to the -75°C freezer until hormone measurements (Naderi *et al*., 2015). After homogenizing the frozen samples by hand held homogenizer in the laboratory, the measurement of estradiol and testosterone levels was performed using the DiaMetra hormone detection kit and ELISA device (model ELx808) according to the instructions of the kit manufacturer.

Sex ratio determination

Sampling was performed to determine the sex of the fish at the end of the rearing period, 125 d. Sixty fish (20 from each replication) were sampled from each treatment. Samples were stored in the -20 freezer until checked. The aceto-carmine method (Guerrero III and Shelton, 1974; Wassermann and Afonso, 2002) was used to determine the sex of fish. Briefly, after removing the gonad from the fish's abdomen, parts of the gonad were placed on a slide, and after adding a drop of aceto-carmine diluted with acetic acid (1:10), a slide was placed on it and crushed by pressing the gonad tissue, and then viewed under a light microscope (Weber *et al*., 2019).

Data analysis

Data normality and homogeneity of variance were tested using the Kolmogorov-Smirnov and Leven's test and then experimental data were analyzed by one-way analysis of variance ANOVA. The Tukey multiple range test was used to compare means. All statistical analyzes were performed using SPSS 22 software. Results

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represented as mean±standard error (SE). The significance level was considered 0.05 (Tan *et al*., 2020).

Results

Sex reversal

In all treatments, a significant difference was observed with the control treatment (p<0.05), which indicates the positive effect of this drug on sex reversal in rainbow trout (Fig. 1). However, no significant difference was observed between the LET or LET-CS treatments.

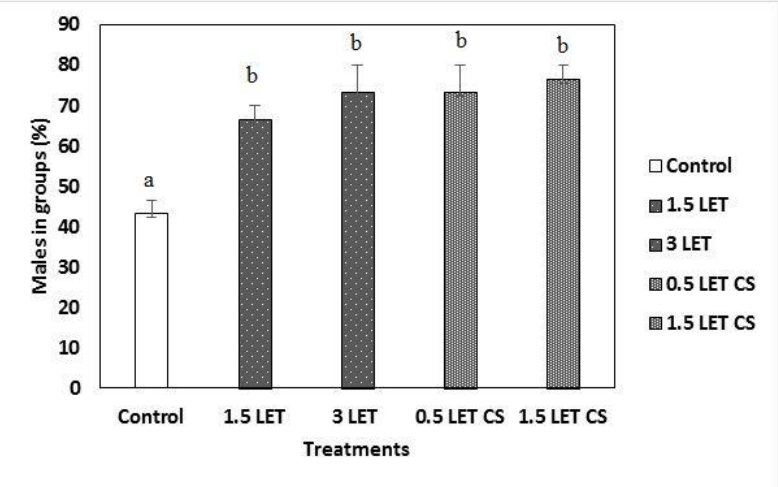

Figure 1: Percentage of masculinization in different treatments. Different letters indicate a significant difference between treatments

Sex steroid results

The results of the measurement of the testosterone levels are presented in Table 1. According to the data, in d 30, a significant increase was observed in 1.5 mg LET-CS treatment (p<0.05). While, in day 60, 3 mg LET and 1.5 mg LET-CS treatments showed a significant increase (p<0.05), however, there was no significant difference between two mentioned treatments. The amount of estradiol was detected only in the control treatment, and in the rest of the treatments, the amount of this hormone for the hormone detection kit was not detectable.

Table 1: Testosterone and estradiol levels (ng/mg) in different treatments (mean ± SD). Different letters in each column indicate a significant difference between treatments. ND: not detected

	Testosterone		Estradiol	
	Day 30	**Day 60**	**Day 30**	**Day 60**
Control	33.10±1.5^{a}	31.81±0.5^{a}	808.53	895.63
1.5 LET	32.89±1.06^{a}	35.80±2.2^{a}	ND	ND
3 LET	34.94±1.74^{a}	49.89±3.41^{b}	ND	ND
0.5 LET-CS	36.01±1.72^{a}	41.19±2.01^{a}	ND	ND
1.5 LET-CS	43.09±3.02^{b}	54.94±3.38^{b}	ND	ND

Mortality

The mortality in period of drug therapy (60 d) in all treatments was significantly higher than the control and in LET-CS treatments was significantly higher than LET treatments (p<0.05) (Table 2). On

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the other hand, the percentage of mortality in the post-drug therapy period (61-125 d) was significantly different only in LET-CS treatments compared to control and other treatments (p<0.05). In general, the highest percentage of mortality was observed in LET-CS groups. According to field observation, larvae fed with LET-CS had less appetite to food.

Table 2: Percentage of mortality (%) during the breeding period (mean ± SD). Different letters indicate a significant difference between treatments.

	Control	1.5 LET	3 LET	0.5 LET-CS	1.5 LET-CS
Day 1-60	29.83 ± 1.11 [a]	40.93 ± 1.19 [b]	41.13 ± 0.99 [b]	47.76 ± 2.97 [c]	50.43 ± 2.24 [c]
Day 61-125	9.45 ± 0.35 [a]	11.65 ± 0.95 [a]	12.08 ± 0.48 [a]	15.69 ± 1.7 [b]	16.56 ± 1.09 [b]
Total	27.03 ± 0.88 [a]	36.73 ± 1.92 [b]	36.9 ± 0.94 [b]	43.33 ± 2.87 [c]	45.8 ±1.49 [c]

Weight gain

Weight gain of fry during the period of drug therapy in all treatments was significantly higher than the control treatment and in LET-CS treatments was higher than LET treatments (p<0.05) (Table 3). But in the period of post-drug therapy, weight gain only in LET-CS treatments was significantly more than the control treatment (p<0.05).

Table 3: Weight of fry (g) during the experiment (mean±SD). Different letters indicate a significant difference between treatments.

	Control	1.5 LET	3 LET	0.5 LET-CS	1.5 LET-CS
Day 1-60	0.747 ± 0.09 [a]	0.787 ± 0.01[b]	0.797 ± 0.01[b]	0.827 ± 0.01[b]	0.827 ± 0.01[b]
Day 61-125	7.427 ± 0.18[ab]	7.036 ± 0.17[a]	7.64 ± 0.21[ab]	7.817 ± 0.58[ab]	8.37 ± 0.20[b]
Total	8.17 ± 0.12 [ab]	7.83 ± 0.18[a]	8.84 ± 0.22[ab]	8.64 ± 0.59[ab]	9.197 ± 0.19[b]

Discussion

Achieving solutions that increase production efficiency as well as high profitability is one of the important goals of the aquaculture industry. Sex reversal is one of the most basic methods that in addition to increasing the quality of production reduces production costs (Budd *et al.*, 2015). The gonadal phenotype of fish can be altered regardless of genetic sex by the use of sex steroids or drugs that inhibit the production of natural fish steroids (Tsai and Wang, 1997; Naderi *et al.*, 2015).

The results showed that all treatments had a positive effect on increasing the percentage of males and LET could cause sex reversal in rainbow trout. However, sex reversal in none of the treatments was performed completely. Xu *et al.* (2021) used MT and LET at doses of 1.5 and 50 mg/kg of food, respectively, to investigate the mechanism of gonadal formation in all-female triploid rainbow trout (XXX). Due to the tendency of these fish to masculinization in normal conditions, the effect of MT and LET on gonadal growth and expression of male-specific genes was investigated. Their results showed that these two drugs did not affect ovarian development in the first stage (56-80 days after fertilization), but in the second stage (94-777 days after

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fertilization) LET performed better than MT on gonadal morphology, the expression of male-specific genes and decreased expression of female genes. Xu *et al*. (2021) used much higher dose that affected only in 56-80 days after fertilization. Although, it should be mentioned that triploid (XXX) fish, were used in this research. It seems to determine the appropriate dose of masculinization of rainbow trout with LET, more research is needed. But the results of present study also show that the drug dose is not the only determining factor. As inferred from the studies, the method of drug administration and the time of starting the drug, such as the immersion method before starting active feeding, may have a greater effect on the success rate of masculinization in this fish than increasing the dose of the drug, as Weber *et al*. (2019) performed immersion one week after fertilization and before the start of active feeding.

It is noteworthy that despite the doubling of the dose of LET compared to LET-CS treatments, the percentage of masculinization was not significantly different between them and it can be deduced that chitosan as a drug carrier was quite effective in increasing the effect of the drug. The results of the amount of testosterone and estradiol in treatments also confirm this presumption, so that on the 30th d, despite using half of dose, only the 1.5 mg LET-CS treatment showed a significant increase in testosterone (Table 2). Injection of LET into rainbow trout (Akbary *et al*., 2015) and fadrozole into coho salmon, *Oncorhynchus kisutch*, (Afonso *et al*., 2000) increased testosterone levels in fish. Wisdom *et al*. (2018) also reported that injection of nanochitosan-binding aromatase inhibitors (LET and fadrozole) had a greater effect on increasing testosterone levels in walking catfish, *Clarias magur* than aromatase inhibitor alone.

The results of the present study showed that LET and LET-CS treatments both caused to increase mortality during the period of drug therapy. On the other hand, LET-CS treatment had higher mortality than LET treatment, which shows that the drug alone and in combination with nanochitosan had a negative effect on larval survival. The process of binding the drug to chitosan nanoparticles involved using chemicals such as acetic acid (Calvo *et al*., 1997; Gomathi *et al*., 2017; Wisdom *et al*., 2018), which can change the taste/odor of fish food and therefore, it would be responsible of reduced fish appetite and thus increase the observed losses. Different environmental factors, diet characteristics, and odors can affect the taste system of fish. The taste system of fishes highly species-speciifc (Kasumyan, 2019). Out of 37 fish species investigated for their taste preferences to the diet containing citric or other acids, 16 species showed a high decrease in food acceptance (Kasumyan and Døving, 2003). Another factor that can increase the loss of all treatments in the early days of the project was the water pollution of the hatchery due to 4 days raining in the early days of the study. The resulting mudflow may have

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exacerbated the stress created in the masculinization process in the experimental groups and resulted in more mortality, however we need more experimental data. Oral administration of LET and MT had no significant effect on survival of yellow catfish, *Pelteobagrus fulvidraco* (Shen *et al*., 2015).

The results showed that LET slightly increased the weight gain at the end of the study period (day 125) in the treatments. At the same time, a slight but significant increase in the weight gain of all treatments compared to the control was observed during the period of drug therapy (d 1-60). Qin *et al*. (2020) reported that LET has no significant effect on the growth of *Nibea mitsukurii* and *N. albiflora*. However, Shen *et al*. (2015) indicated that lower doses of LET promoted the growth of yellow catfish, *Pelteobagrus fulvidraco*, and higher doses had no effect on growth. Given that maximum mortality and weight gain were observed in the LET-CS group, the growth data appear to be inconsistent. Since the highest mortality rate was observed in the mentioned group, reducing the larval density could create better conditions (better water quality, reducing the larvae's competition for food, oxygen and space) for the remaining larvae, and therefore this group experienced better growth efficiency (North *et al*., 2006).

Based on the results, it is concluded that the use of a combination of chitosan nanoparticles for the administration of LET can significantly reduce estradiol and increase testosterone and thus the success of masculinization. Therefore, nanoparticles can be used instead of LET or possibly any other aromatase inhibitor alone to increase the efficacy of the drug and prevent the use of large amounts of the drug and its leakage into the environment.

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