



Nanotechnological innovation for the production of daughter less Tilapia, *Oreochromis niloticus* (Linnaeus, 1758)

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Received: November 18, 2016; Revised received: April 30, 2017; Accepted: September 10, 2017

Abstract: The aim of present work was to develop a new Fadrozole (FDZ)-loaded Poly(D,L-lactide-co- glycolide) lactide:glycolide (50:50)(PLGA) nanoparticles for effective delivery of the masculinization drug, Fadrozole, as an alternative to commercially available masculinization agents like testosterone (dietary supplementation of 17 α -methyltestosterone) which are steroids and banned in most EU countries. The FDZ-loaded PLGA NPs were prepared by solvent displacement technique. The particle size of FDZ-loaded PLGA NPs was analyzed using LICOMP particle size analyser. It was found to be in the range of 60 \pm 66.7 nm to 560 \pm 66.7 nm with average size of 201.4 \pm 66.7 nm, where the Zeta potential was estimated to be about -20.82 mV, a series of experiments were carried out to induce masculinization using FDZ-loaded PLGA nanoparticles during the sex differentiation period. Tilapia, *Oreochromis niloticus* fry were treated with FTZ-loaded PLGA nanoparticles at dosages 5, 25, 50 and 100ppm/kg diet for 10, 15 and 30 days. The results indicated an increase in the proportion of males with dosage and duration of treatment. The male percentage was 92.35 \pm 0.86 for T7(50 ppm) at 10 days, 97.76 \pm 1.12 for T7 (100 ppm) at 15 days and 100 % for both T6 (50ppm) and T7 (100 ppm) at 30 days. This is first time done by using nanotechnology efficiently in Tilapia species which is very important Fresh water aquaculture species in present era. Which showed increase the male population with lesser dose of nano-encapsulated Fadrozole (FDZ)-loaded PLGA nanoparticles drug as compared with naked control Fadrozole (FDZ) drug delivery.

Keywords: Bio-dégradable, Fadrozole, Masculinisation, Nanoparticle, PLGA , *Oreochromis niloticus*

INTRODUCTION

Tilapia has been exacting as the Food Fish of the 21st century and is widely known as “Aquatic chicken” (Khawa *et al.*, 2003). The culture of only male tilapia or hybrid is only allowed in India so as to evade / detention productive breeding during culture period (NABARD, 2016).

Nanoparticles (NPs) are believed to be the best drug delivery system, have substantial potential for drug directing means target drug delivery and show numerous benefits over conformist delivery systems like low drug requirement, specificity, target drug delivery etc. (Otilia *et al.*, 2005, Hughes, G.A., 2005). Drugs conjugated with nanoparticles can be targeted to a specific site of action with accompaniment decrease in the extent of drug necessary and amount venomousness means toxicity, permitting safe delivery of deadly therapeutic drugs as well as defence of non-goal matters and cells from unadorned side effects and indemnities (Brannon-Peppas, L., 1995). Since the application of

nanoparticle is always associated with the concern of its ill effect, recyclable polymeric nanoparticles obligate anew been the choice of the researchers. These polymers have already been used as carriers for anti-cancer agents (Brigger *et al.*, 2002), (Vauthier *et al.*, 2003) With this background, the present research work has been designed to develop PLGA loaded fadrozole nanoparticles for producing all male population of tilapia *Oreochromis niloticus*.

Only in the later half of the 20th century, use of gonadal hormones in the manipulation of sex was initiated and the initial studies were mainly carried out on fishes having little or no commercial significance. In fish, two aromatase iso-forms have been designated, one specially articulated in the ovary (implicit by the CYP19a gene) and one specially expressed in the brain (coded by the CYP19b gene) (Chiang *et al.*, 2001). Tchoudakova *et al.*, (2011) stated that the gonads, aromatase takes revealed to be a key enzyme adaptable sexual diversity in fish. During early progress, obstructive aromatase will distinguish the gonads into testis

(Komatsu *et al.*, 2006), while estrogen administration will cause the development of ovaries (Piferrer, F., 2001). In the recent past decade the Fadrozole a non-steroidal aromatase inhibitor has been broadly used to operate the sex ratio and its effects on gonadal development in Nile tilapia (Kwon *et al.*, 2000) (Kwon *et al.*, 2001) (Kwon *et al.*, 2002), common carp (Pandian, T. J. and Varadaraj, K., 1987), etc. Fadrozole inhibiting the aromatase enzyme by quickly joining to the heme of the cytochrome P450 subunit of the enzyme, resulting in a reducing of estrogen biosynthesis in all tissues that can result in an elevated level of androgen (by preventing the conversion of androgens to estrogens), thus leading sex reversal from genetic female to phenotypic male. In India, Das *et al.*, 2007 did series of experiment and he got 100% male population in *O. mossambicus* by administering 17α MT through rearing medium for 7 to 10 days old fry for a period of 11 to 19 days at dosage of 5 and 10 $\mu\text{g/l}$ water. It is possible to manipulate the sex ratio by letrozole (a non-steroidal aromatase inhibitor) in the *O. mossambicus* by oral administration. This is first time done by using nanotechnology efficiently in the Nile Tilapia. Which showed increase the male population with lesser dose of nano-encapsulated Fadrozole (FDZ)-loaded PLGA nanoparticles drug as compared with naked control Fadrozole (FDZ) drug delivery.

MATERIALS AND METHODS

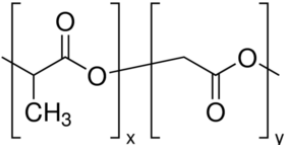
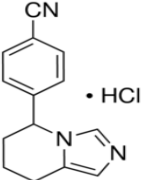
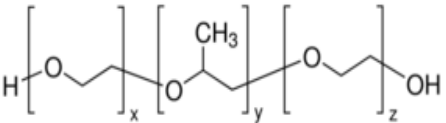
Chemicals used

Preparation of FDZ-loaded PLGA NPs: The nanoparticles loaded with Fadrozole were prepared by solvent displacement technique as per the reported meth-

od (Mondal *et al.*, 2008) with some alteration. Briefly, a solution of 150mg of PLGA (50:50) in 10ml of acetone taken into a Tronssterile centrifuge tube conical bottom of 50ml capacity and did vortexing using vortex mixture. After that another Tarsons sterile Centrifuge Tube Conical Bottom of 50ml capacity containing 50mg of Fadrozole (drug polymer ratio 1:3) taken into the 20ml of acetone with vortexing using vortex mixture. This prepared vortex mixture of both chemicals added together in another sterile Centrifuge Tube Conical Bottom of 50ml capacity and vortex it for 5 min. This newly prepared mixture of PLGA and Fadrozole was added at a constant flow rate (0.2 ml/min) to 1 lt. capacity beaker which contain 500 ml an aqueous phase containing 1% (w/v) poloxamer-188 at a under mechanical stirring at 10,000 rpm. The organic phase was evaporated at room temperature with constant stirring for 5 h. Finally the nanoparticles were isolated by centrifugation at 17500 rpm 4°C for 40 min.

Characterization of nanoparticles: Measurement of size with zeta potential was carried out by using Horiba nano Partica SZ-100 series (Japan) which is based on dynamic light scattering (DLS) technique. Depending on the physical properties of the sample, the dynamic range of the equipment was 0.3 nm to 8 μm .

Analysis of the charge on the surface or zeta potential of particles is performed by injecting the sample into a disposable cell and a measurement of the Electrophoretic mobility. This calculates zeta potential. The zeta potential of the sample is most often used as an indicator of dispersion stability. Large magnitude zeta potential values indicate that an electrostatically stabilized suspension will remain stable. The surface morpholo-

Chemical Name	Chemical Formula / Structure	Manufa-cturing Firm	Toxicity
Poly(D,L-lactide-co-glycolide) lactide:glycolide (50:50), mol wt 30,000 - 60,000,		SIGMA-ALDRICH	Nil
Fadrozole hydrochloride (4-(5,6,7,8-Tetrahydroimidazo[1,5-a]pyridin-5-yl)-benzonitrile) $\geq 98\%$ (HPLC)		SIGMA-ALDRICH	Nil
Poloxamer 188 (Kolliphor® P 188)		SIGMA-ALDRICH	Nil
Ethylene glycol anhydrous, 99.8%	HOCH ₂ CH ₂ OH	SIGMA-ALDRICH	Nil
Other Chemicals and Solvents (Analytical grade)	-----	Merck India	Nil

(Chemical Formula / Structure Source: Sigma Catalogue 2016-17)

Table 1. Male, female and survival percentage of different treatments at different time interval.

Treatment	10 days	15 days	30 days						
	Male (%)	Female (%)	Survival (%)	Male(%)	Female (%)	Survival (%)	Male(%)	Female (%)	Survival (%)
T1	48.48 ^a ±1.51	51.51 ^c ±1.51	85.92±3.72	47.15 ^a ±0.51	52.84 ^c ±0.51	94.28±1.64	47.40 ^a ±0.78	52.59 ^c ±0.78	96.66±5.84
T2	65.87 ^c ±0.79	34.12 ^d ±0.79	85.92±4.54	70.05 ^c ±0.77	29.94 ^c ±0.77	92.38±2.43	88.96 ^b ±1.69	11.03 ^b ±1.69	100.00±3.84
T3	48.45 ^a ±0.90	51.54 ^c ±0.90	85.18±4.63	47.25 ^a ±0.54	52.74 ^c ±0.54	94.28±0.12	47.24 ^a ±0.94	52.75 ^c ±0.94	96.66±6.14
T4	55.08 ^b ±0.40	44.91 ^c ±0.40	89.62±1.91	58.37 ^b ±0.65	41.62 ^d ±0.65	93.33±1.52	89.31 ^b ±1.97	10.68 ^b ±1.97	98.88±4.01
T5	78.39 ^d ±1.42	21.60 ^e ±1.42	87.40±4.14	85.21 ^d ±1.23	14.78 ^b ±1.23	92.38±1.62	95.92 ^c ±2.09	4.08 ^a ±2.09	97.77±1.11
T6	88.76 ^e ±1.07	11.23 ^b ±1.07	85.18±3.22	95.53 ^e ±0.89	4.46 ^a ±0.89	93.33±1.44	100.00 ^a	0.00 ^a	97.77±4.85
T7	92.35 ^f ±0.86	7.64 ^a ±0.86	85.18±5.10	97.76 ^e ±1.12	2.23 ^a ±1.12	92.38±1.62	100.00 ^a	0.00 ^a	95.55±6.75
P value	<0.001	<0.001	0.985	<0.001	<0.001	0.985	<0.001	<0.001	0.985

Table 2. The particle size, zeta-potential and poly dispersive index a PLGA and FDZ-loaded PLGA NPs.

Nanoparticles	Particle size (nm)	Zeta potential	Poly dispersive index / normal Standard Deviation
PLGA	136±74.7	-18.20 mv	0.547
FDZ- PLGA	201.4±66.7	-20.82 mv	0.331

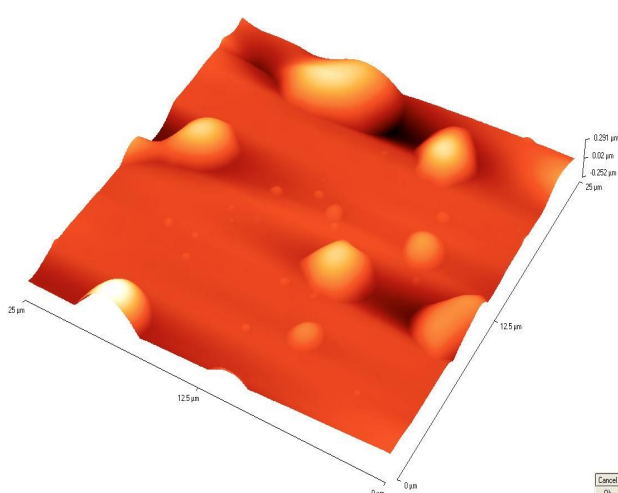


Fig. 1. Three-Dimension AFM image of FDZ-loaded PLGA NPs.

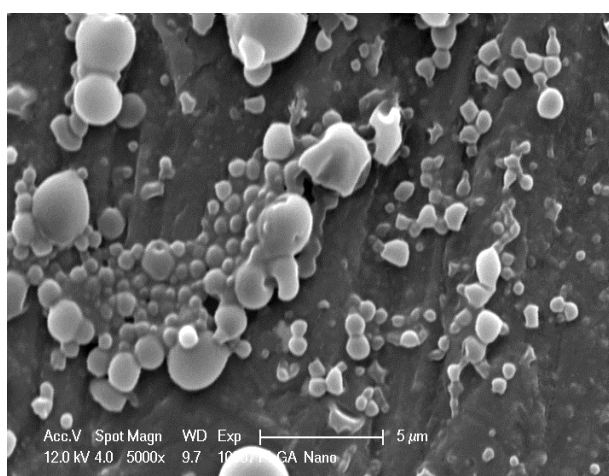


Fig. 2. SEM image of FDZ-loaded PLGA NPs.

gy (roundness, smoothness and formation of aggregates) was studied by Atomic Force Microscopy and scanning electron microscope.

Preparation of diet: The treatment diet used in present study was the Firest Bite. Which is commercially available hatchling and fry feed procured from Hikari, Japan. About 100g of feed was weighed and used for incorporation of PLGA conjugated Fedrazole.

Incorporation of FDZ-loaded PLGA NPs into fish feed: PLGA NPs conjugated FDZ were incorporated into the feed as follows: The appropriate amount of FDZ-loaded PLGA NPs i.e. 5, 25, 50 and 100 mg (weighed accurately using an electrical balance) was dissolved in 50ml of 95% acetone. The required quantity of powdered feed was taken in an enamel tray and the FDZ-loaded PLGA NPs dissolved in acetone were sprayed uniformly using a chromatogram column sprayer.

Preparation of negative control: The negative control diet was prepared in the same manner using only the solvent (acetone).

Preparation of positive control: The positive control diet was prepared in the same manner using only 100mg pure Fadrozole in the solvent (acetone).

Incorporation of PLGA NPs into fish feed: Incorporation of PLGA nanoparticles into the fish feed was accomplished as follows: The appropriate amount of PLGA nanoparticles that is 100 mg (weighed accurately using an electrical balance) was dissolved in 50ml of 95% acetone. The 10 gm of powdered feed was taken in an enamel tray and the PLGA nanoparticles dissolved acetone was sprayed uniformly using a chromatogram column sprayer.

Development of brood-stock: The brooders were reared in cemented tank (6×4×3ft) until they are matured. The gravid female and male tilapia were released in aquaria (2×1×1ft) in the ratio of 2:1 (F: M).

The fish were fed twice daily on artificial diet as well as mosquito larvae, tubifex, bloodworm, earth worm and zooplankton at libido.

Seed production of tilapia: The female and male brooders were placed in a big aquarium with muddy bottom to released eggs and sperm. After fertilization the female takes all eggs into her mouth for the incubation upto free swim. The resultant fry were used for experimentation. The aquaria were aerated using an air blower.

FDZ-loaded PLGA NPs administration to fry

Trial 1: This trial was conducted for 10 days. The two-day-old spawn (initial weight 6.13mg) were divided into 7 groups of 35 each and designated as T₁, T₂, T₃, T₄, T₅, T₆ and T₇ for negative control, positive control, PLGA nanoparticle (100 ppm) and 5, 25, 50 and 100ppm of FDZ-loaded PLGA NPs respectively. Fishes were fed daily (100% body weight) for the first ten days.

Trial 2: This trial was conducted for 15 days. The one-day-old spawn (initial weight 3.98mg) were divided into 7 groups of 30 each and designated as T₁, T₂, T₃, T₄, T₅, T₆ and T₇ for negative control, positive control, PLGA nanoparticle (100 ppm) and 5, 25, 50 and 100ppm of FDZ-loaded PLGA NPs respectively. They were fed daily @ 100% body weight for the first ten day and 75% for the next 5 days (11th and 15th day).

Trial 3: This trial was conducted for 30 days. The two-day-old spawn (initial weight 4.0mg) were divided into 7 groups of 45 each and designated as T₁, T₂, T₃, T₄, T₅, T₆ and T₇ for negative control, positive control (100ppm pure Fadrozole without nanoparticles), PLGA nanoparticle (100 ppm) and 5, 25, 50 and 100ppm of FDZ-loaded PLGA NPs respectively. They were provided feed daily @ 100% body weight for the first ten days, 75% for the next 15 days and 50% for the last 5 days.

Evaluation of all treatments: On termination of the experiments, all the surviving fish were harvested, counted and measured for weight and length and sexed by simple light macroscopic inspection. The phenotypic sex of the treated and control fish was determined with the help of secondary sexual characters, such as the shape of the belly, presence of number of gonopore.

Statistical analysis: Data on percentage of male produced in each treatment was analyzed using one way ANOVA to test the significant difference of mean values and standard error using SPSS 18 software.

RESULTS AND DISCUSSION

Physiochemical characterization of nanoparticles: In the present study, the Fadrozole loaded PLGA Nanoparticles (FDZ-PLGA NPs) were prepared by using solvent displacement technique as per the reported method with some alteration had particle size be in the range of 60±66.7 nm to 560±66.7 nm with an average size of 201.4±66.7 nm and zeta potency about -20.82mV (Table 2, Fig. 1, 2). Three trials were made to test the efficiency of FDZ-PLGA NPs to induce the masculinization in research animal. FDZ-loaded PLGA NPs were prepared by

solvent displacement technique. This yielded spherical powder particles with diameter of LTZ-loaded PLGA nanoparticles 15–100nm. The loading efficiency as well as drug entrapment efficiency of LTZ-loaded PLGA NPs was 77.44±0.24 and 43.03±2.20%, respectively. The zeta potential values of LTZ-loaded PLGA NPs were found to be negative, -12 to -19.50 mV (Mondal *et al.*, 2010).

The dexamethazone in PLGA nanoparticles was developed for treating disease like rheumatoid, (Park *et al.*, 2012) the average size was 50 nm with drug loading efficiency was 56.18±5.39 %.

This is justified that the developed nanoparticle size occurred in between the reported nanoparticles size which is cited above and particles are morphologically spherical and compact and shown more stable.

Male and female percentage and survival rate: Generally, Fadrozole shows effects on sex ratio, increases in percentage of male production with directly proportional to the dose of FDZ-loaded PLGA NPs. (Tables 1). The survival percentage of fry (Tables 1) observed that there was no mortality observed in the FDZ-loaded PLGA NPs fed experimental fishes. It is indicated that the use of FDZ-loaded PLGA nanoparticles in *Tilapia (Oreochromis niloticus)* is reasonably safe for masculinization of fish.

The male percentage was 92.35±0.86 for T₇ at 10 days, 97.76±1.12 for T₇ at 15 days and 100 % for both T₆ (50ppm) and T₇ (100 ppm) at 30 days, Lowest male percentage was found at T₄ treatment of all groups. It was reported that the male percentage of treatment T₂ (naked fadrozole at 100 ppm) and Treatment T₄ (FDZ-PLGA NPs at 5 ppm) were 88.96 % and 89.31 % respectively for 30 days treatment (Trial 3). These finding shows that FDZ in nano forms can reduce 90% of prescribed dose of fadrozole for masculinization of commercial fish like tilapia.

It was observed that the percentage of male production in treatment T₄ during 10 and 30 days' time interval was increased from 55.08 to 89.31 %. It is 'therefore' administered FDZ- PLGA NPs at low dose (5 ppm per kg) for prolong period, presented significant sex reversal and also helped to reduce cost of drug and its adverse impact on *Oreochromis niloticus*. The sex ratio was significantly (P<0.001) altered in the FDZ-PLGA NPs treated groups in compare to control groups in all treatments with respect to trials and doses except T₃ which has PLGA nanoparticle without Fadrozole (drug) and check the impact of nanoparticle on experimental fishes (Table 1). Among LTZ-loaded PLGA nanoparticles treated groups, the lowest percentage of males (82.63%) was observed in 5ppm group; while the highest percentage of males (100%) was found at 50 and 100 ppm when fed for 30 days. Das (2007) observed that the manipulation of the sex ratio in *O. mossambicus* was possible by oral administration of letrozole (a non-steroidal aromatase inhibitor). Dietary administration of letrozole (100 and 200 ppm) was produced 92-100% males when fed to 5-6 day-old-fry for 30 days.

In the recent past decade the Fadrozole a non-steroidal aromatase inhibitor has been broadly used to operate the sex ratio and its effects on gonadal development in Nile tilapia (Kwon *et al.*, 2000) (Kwon *et al.*, 2001) (Kwon *et al.*, 2002), common carp (Pandian, T.J and Varadaraj, K., 1987), etc. Fadrozole inhibiting the aromatase enzyme by quickly joining to the heme of the cytochrome P450 sub-unit of the enzyme, resulting in a reducing of estrogen biosynthesis in all tissues that can result in an elevated level of androgen (by preventing the conversion of androgens to estrogens), thus leading sex reversal from genetic female to phenotypic male. In India, 100% male population in *O. mossambicus* by administering 17 α MT through rearing medium to 7 or 10 days old fry for a period of 11-19 days at dosage of 5 and 10 μ g/ l water. (Das *et al.*, 2007) it is possible to manipulate the sex ratio by letrozole (a non-steroidal aromatase inhibitor) in the *O. mossambicus* by oral administration. It is observed that LTZ-loaded PLGA nanoparticles treated groups, the lowest percentage of males (82.63%) was observed in 5ppm group; while the highest percentage of males (100%) was found at 50 and 100 ppm when fed for 30 days (Joshi *et al.*, 2015). The results showed an increase in the proportion of males with dosage and duration of treatment and cited reported works supported the present work.

Conclusion

The results stated developed a new Fadrozole (FDZ)-loaded PLGA nanoparticles for effective delivery of the masculinization drug. The use of nanoparticles significantly showed the effect on masculinization as compared with naked drug delivery. This type of Nano encapsulated drug can be prepared easily and economically by using standard protocol at farm level lab by using only magnetic stirrer with basic lab equipment.

ACKNOWLEDGEMENTS

We are thankful to the CIFE, Mumbai and ICAR, New Delhi for financial support.

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