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The effects of COX-Inhibitors (Diclofenac and Ibuprofen) on growth rate, mortality and sex reversal in Nile Tilapia (*Oreochromis niloticus*)

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Abstract. Several studies have been conducted to detect the direct effect of inhibiting the aromatase activity, the rate limiting enzyme that converts androgens to estrogens needed for ovarian differentiation in fish to overcome the immediate need for a more environmentally friendly substitute of methyl testosterone. Cyclooxygenase (COX)-inhibitors are potent and irreversible inhibitors of the COX pathway and since studies on human breast cancer cells shows that they decrease aromatase messenger ribonucleic acid (mRNA) expression at the transcriptional level we tested the hypothesis of possible aromatase inhibition by the non-selective COX-inhibitors in fry fish tilapia. The effects of supplementation of COX-inhibitors (diclofenac and ibuprofen) in the diets of tilapia on growth rate, mortality and sexual differentiation have been studied. 20 Genetically females (XX) (*O. niloticus*) larvae were stocked in triplicates in a closed system and each were given control diet (C group) and control diet supplemented with (10 $mgKg^{-1}$) diclofenac (1% diclofenac group), (5 $mgKg^{-1}$) ibuprofen (0.5% ibuprofen group), and (5 $mgKg^{-1}$) (0.5% diclofenac group) respectively for 4 weeks. After the 4^{th} week all diets were changed to control diet. At the end of 12-weeks, no significant differences were found in growth rate (GR) between diets ($p>0.05$). Mortality ranged from 1.67% +- 2.89 (SD, n=3) in control group to 58.3% +- 14.4 (SD, n=3) in the 1% diclofenac group during the experimental feeding and from 6.67+-2.89 (SD, n=3) to 63.3%+-10.4 (SD, n=3) at the end of 12-weeks period. 7% In the control group, 36% in the 1% diclofenac group, 17% in the 0.5% ibuprofen group, and 22.2% in the 0.5% diclofenac group respectively never produced egg during the entire experimental period. Macroscopically all the non-spawning fish in the experimental groups were females with apparently larger ovaries and full of eggs compared to control. Microscopically they were full of apparently normal eggs with morphology similar to those of control. Postulated mechanisms of action of the supplemented COX-inhibitors are discussed. Based on the above it can be concluded that the use of COX-Inhibitors during the crucial period could modulate aromatase activity and affect reproduction in Nile tilapia.
Key Words: COX-inhibitors, Tilapia, aromatase, sexual differentiation.

Resumen. Varios estudios se han llevado a cabo para detectar el efecto directo de inhibir la actividad aromatasa, la enzima que convierte los andrógenos a los estrógenos necesarios para la diferenciación ovárica en pescados para superar la necesidad inmediata de un sustituto más amigable al medio ambiente que la metiltestosterona. Ciclooxigenasa, inhibidores de la (COX) - son inhibidores potentes e irreversibles de la via de ciclooxigenasa (COX). Ya que los estudios en las células cancerosas humanas de mama demuestran que disminuyen la expresión del ácido ribonucleico ARN mensajero del aromatasa (ARNm) a nivel transcripcional, hemos probado la hipótesis de la inhibición posible del aromatasa por los inhibidores no selectivos de la COX en Tilapia. Los efectos de la suplementación de inhibidores de la (COX) (diclofenac e ibuprofen) en las dietas de la Tilapia sobre tasa de crecimiento, mortalidad y la diferenciación sexual se han estudiado. 20 genéticos larvas de las hembras (XX) (*O. niloticus*) fueron sembradas por triplicado en un sistema cerrado y cada uno fue dado la dieta del control (grupo c) y la dieta del control suplementado con el diclofenac (10 mgKg-1) (grupo del diclofenac 1%), el ibuprofen (5 mgKg-1) (grupo del ibuprofen 0.5%), y (5 mgKg-1) (grupo del diclofenac 0.5%) respectivamente durante 4 semanas. Después de la 4^a semana todas las dietas fueron cambiadas a la dieta del control. Al final de 12 semanas, no se encontró ninguna diferencia significativa en la tasa de crecimiento entre las dietas ($p> 0.05$). La mortalidad osciló entre 1.67% + - 2.89 (SD, n=3) (grupo de control) a 58.3% + - 14.4 (SD, n=3) (grupo del diclofenac 1%) durante la alimentación experimental y de 6.67+-2.89 (SD, n=3) a 63.3%+-10.4 (SD, n=3) al fin de 12 semanas. El 7% en grupo de control, el 36% en grupo del diclofenac 1%, el 17% en grupos del ibuprofen 0.5%, y 22.2% en grupos del diclofenac 0.5% nunca produjeron huevos respectivamente durante el período experimental entero. Macroscópico todos los pescados que no desove en los grupos experimentales eran hembras con ovarios al parecer más grandes

y lleno de huevos comparado al control. Visto en microscópico llenos de huevos al parecer normales con la morfología similar a las del control. Los mecanismos postulados de la acción de los inhibidores suplementados se discuten. De acuerdo con lo anterior llegamos a la conclusión que durante el período crucial usar los inhibidores de la COX podría modular la actividad de la aromatasa y afectar la reproducción de la Tilapia del Nilo.
Palabras clave: inhibidores de la COX, Tilapia, aromatasa, diferenciación sexual.

Abstract. Câteva studii au fost effectuate pentru detectarea unor inhibitori direcți ai activității aromatazei, enzima care limitează rata conversiei androgenilor în estrogeni necesari diferențierii ovariene la pești, pentru a rezolva necesitatea iminentă a unui substituent al metiltestosteronului, mai prietenos mediului. Inhibitorii ciclooxigenazei (COX) sunt inhibitori puternici și ireversibili ai căii COX și având în vedere că studiile pe cellule cancerose mamare de om arată că ei scad nivelul expresiei ARNm la nivel transcripțional, am testat ipoteza posibilei inhibiții aromatazice prin inhibitori neselectivi ai COX la alevinii de tilapia. S-a urmărit efectul suplimentării hranei tilapiei cu inhibitori ai COX (diclofenac și ibuprofen) asupra ratei de creștere, mortalității și diferențierii sexuale. Douăzeci de alevini genetic femeli (XX) (*O. niloticus*) au fost crescuți în loturi experimentale (triplicates) în sistem recirculant închis. Peștii au fost hrăniți cu hrană martor, hrană suplimentată cu diclofenac 1%, ibuprofen 0,5% și diclofenac 0,5%, timp de patru săptămâni. Dupa cea de a patra săptămână, dieta suplimentată a fost schimbată cu dieta martor. Dupa 12 săptămâni, nu s-au găsit diferențe semnificative în cea ce privește rata de creștere ($p>0,05$). Mortalitatea a variat în intervalul 1,67% +- 2,89 (SD, n=3) în cazul grupului martor, 58,3% +- 14,4 (SD, n=3) în lotul diclofenac 1%, pe durata experimentului, iar după cele 12 săptămâni 6,67+- 2,89 (SD, n=3) până la 63,3%+-10,4 (SD, n=3). Nu au produs icre deloc de-a lungul întregii durate experimentale: 7% din femele în cazul martorului, 36% în cazul lotului hrănit cu diclofenac 1%, 17% în lotul ibuprofen 0,5%, și respectiv 22,2% în lotul 0,5% diclofenac. Macroscopic, toate femelele din loturile experimentale care nu au depus icre au prezentat ovare mai mari și mai pline cu icre, comparativ cu cele din lotul martor. Microscopic, ele au fost pline cu icre aparent normale, morfologic similare celor din lotul martor. Se discută în lucrare mecanismele de acțiune ale inhibitorilor COX. S-a concluzionat că folosirea lor într-un moment crucial, modulează activitatea aromatazică și afectează reproducerea la tilapia de Nil.
Key Words: inhibitori ai COX, tilapia, aromataza, diferențiere sexuală.

Introduction. One of the continuing researcher's goals is to continue the work of producing a single sex fry and understanding the mechanisms involved in sex determination in order to overcome the immediate need for a more environmentally friendly substitute of methyltestosterone. Several studies have been conducted to detect the direct effect of inhibiting the aromatase activity, the rate limiting enzyme that converts androgens to estrogens needed for ovarian differentiation in fish (D'cotta et al 2001). Studies on human breast cancer cells shows that cyclooxygenase (COX) inhibitors including the non steroidal anti-inflammatory drugs (NSAIDS) and selective COX-2 decrease aromatase mRNA expression at the transcriptional level (Diaz-Cruz 2005). In addition studies suggest a strong association between aromatase (CYP19) genes expression and the expression of cyclooxygenase (COX) genes, in human adipose stromal cells (Richards & Brueggemeier 2003). Expression of ovarian aromatase in Nile tilapia lay between 3 and 4 days post fertilization in both sexes, with levels of expression high in females that play a decisive role in sexual differentiation achieved by down-regulation of the expression of this gene in males (Kwon et al 2001).

Prostaglandin G/H endoperoxide synthase, also known as cyclooxygenase is a key enzyme that catalyzes the conversion of arachidonic acid (ArA) to prostaglandins. When ArA is released from cell membranes by phospholipases mainly PLA2, it can be metabolized by prostaglandin G/H synthase. Prostaglandin G/H synthase is a membrane-bound protein in the endoplasmatic reticulum of prostaglandin forming cells and exhibits two distinct catalytic activities. The cyclooxygenase (COX) component catalyses the oxidation of the fatty acid arachidonic acid (ArA, 20:4n-6) to the intermediate prostaglandin G2 (PGG2). The hydroperoxidase component mediates the reduction of the 15-hydroperoxyl group of PGG2 to the highly unstable prostaglandin endoperoxide PGH2, which is rapidly converted into PGs (PGE2, PGD2, PGF2) and thromboxanes (Simmons et al 2004). Two isoforms have been identified COX-1 and COX-2, the COX-1 subtype is generally considered to be transcribed constitutively and found in all cells, while COX-2 expression is induced by various stimuli and is predominantly involved in pathophysiological processes such as inflammation (Simmons et al 2004). Fishes posses both COX-1 and COX-2 forms (Javing et al 2004) and it have been sequenced in several fishes, (Grosser et al 2002). It has been demonstrated that in fish an inducible COX-2 (Zou et al 1999) exists as well, in addition to a constitutively expressed COX-1 type and

COX-2 both are approximately 65% to 84% homologues to the mammalian isozymes and also they are widely expressed during development (Grosser et al 2002).

Prostaglandins (PGS) play a physiological significance and a role in intracellular signaling in mammals (Cha et al 2005). They also have physiological functions in fish including control of respiratory and cardiovascular output (McKenzie 2001) as they act as endothelium relaxing factors (Miller & Vanhoutte 2000), ovulation and spawning behavior, oocyte maturation (Goetz et al 1989), nervous system function (Mustafa & Srivastava 1989), osmoregulation (Evans et al 2004) and immune functions (Rowley et al 1995). It can also affect the hypothalamus-pituitary-internal (HPI) axis as in humans that play a role in stress responses (Van Anhal et al 2003). They are also implicated in developmental functions such as body plan development in zebra fish, signal cell motility during gastrulation and vascular tube formation (Grosser 2002; Cha et al 2005). The important sites of prostaglandin production are tissue directly involved in fluid and electrolyte regulation such as gills, opercular membranes and kidneys. Other sites include ovaries and testis (Grosser et al 2002).

Nonsteroidal anti-inflammatory drugs (NSAIDS) are potent and irreversible inhibitors of the COX pathway. Some are non selective COX-1 and COX-2 inhibitors like diclofenac and ibuprofen and others are only COX-2 selective inhibitors (Simmons et al 2004).

In this study we will examine the possible effect of non-selective COX inhibitors on aromatase activity in fry fish Tilapia. Our objective is to investigate the effect of NSAID like Diclofenac and Ibuprofen as non selective COX inhibitors on growth rate, mortality and sex reversal in Nile Tilapia (*Oreochromis niloticus).*

Material and Method. Genetically female tilapia (XX) fry aged approximately 3 days post-hatch were produced through the indirect approach of producing monosex fish (Piferrer 2001).

Commercial feed was used as the control diet. Feed composition are shown in Table (1). Diclofenac and Ibuprofen were purchased from Sigma and Alderich.

Table 1

Feed composition and treatments concentrations

Treatment	*Composition*	*Concentration*
Control (C)	91.8% Dry Matter (OM) As percent of OM: 43.5% Crude Protein (CP) 10.9% Crude Lipid (CL) 13.0% Crude Ash (CA) 18.7 kJ o' Gross Energy	
Diclofenac		1 %
Ibuprofen		0.5 %
Diclofenac		0.5 %

At an age of 3 days post-hatching 20 genetically females (XX) *O. niloticus* larvae were stocked in triplicates, into aquariums each with a capacity of ~45L at Aquatic and Aquaculture Lab, Al Quds University where the study was conducted. Treatments included three different experimental diets and one standard diet serving as control (Table 1) with three repeats for each group. All aquariums in the experiment were disconnected from the recirculation system in order to avoid cross-contamination. In order to maintain adequate water quality 10% of each aquarium's water was changed daily. Water temperature was maintained at ~28 C. A constant photoperiod of 12 h light and 12 h dark was maintained. The test diets were prepared by mixing 10g diclofenac, 5g diclofenac and 5g ibuprofen, with 1Kg commercial feed, to achieve (0.5% diclofenac), (1% diclofenac) and (0.5% ibuprofen) concentrations respectively (Table 1). Feeding started on the same day of stocking and fish were fed twice daily. Feed was made into pellets crushed into smaller sizes and spread on the water surface slowly by hand. Feed

was adjusted according to fish weight: at the 1st week 30% of body weight, 2nd week 20%, 3rd week 15% and from the 4th week on 5%. After the 4th week all diets were changed to control diet.

Fish were weighted every 10 days and counted in each aquarium to determine survival rate during 12-weeks period. Individual fish in each aquarium was weighted to the nearest 0.1g using a digital scale. The growth rate (GR) was determined using linear regression: yt = a + bxt, where yt is the average total weight (g) of the fishes at time t and a is the average weight (g) of fishes at the start of the experiment.

At age of 2 months fish were transferred to big aquariums of one cubic meter with open recirculatory system where water temperature was maintained at ~28 C with constant photoperiod of 12 h light and 12 h dark. Rates of water flow was adjusted to maintain oxygen saturation above 80% and ammonia concentration was always less than 0.2 mg/L. Spawning occurrence was followed another 4 months and each spawning fish was transferred to another aquarium. All the non-spawning fish in the three experimental groups including control were killed and sex determined macroscopically by gonad excision and microscopically by the gonad squash method (Guerrero & Shelton 1974).

Results and Discussion. Growth rate**:** At the end of the 12-weeks no significant differences were found in the average weights and growth rates (GR) and ($P>0.05$), between fish in all experimental groups compared to control. It is well known that there are differences in male vs female growth rates in *Oreochromis* species. Monosex production or even incomplete female prevents reproduction and therefore feed energy could be diverted into growth instead of production of unwanted juvenile fish. Results showed no increase or decrease of growth. This result reflects the fact that growth is regulated by several controls including differences in levels of both sex- related hormones and metabolic hormones such as triiodothyronine. The study of (van Anholt et al 2003) showed that acetylsalicylic acid administration to tilapia (*Oreochromis mossambicus*) reduces basal levels of both triiodothyronine (T3) and cortisol significantly which play an important role in growth. Under controlled experimental factors we should take into consideration the altered physiological effects mediated by the inhibition of prostaglandins (PG) synthesis. In addition Tilapia kept in partially closed systems and fed artificial diets have been reported to have a decreased growth after a certain period of time. This may be due to the fact that tilapia needs a continuous supply of their natural food or because of a build-up of growth inhibiting factors in the system (Jackson et al 1982).

Mortality: Mortality ranged from 1.67%+-2.89 (SD, n=3) in (control group) to 58.3%+-14.4 (SD, n3) in the (1% diclofenac group) during the experimental feeding and from 6.67+-2.89 (SD, n=3) to 63.3%+-10.4 (SD,n=3) at the end of 12-weeks period (see Figure 1).

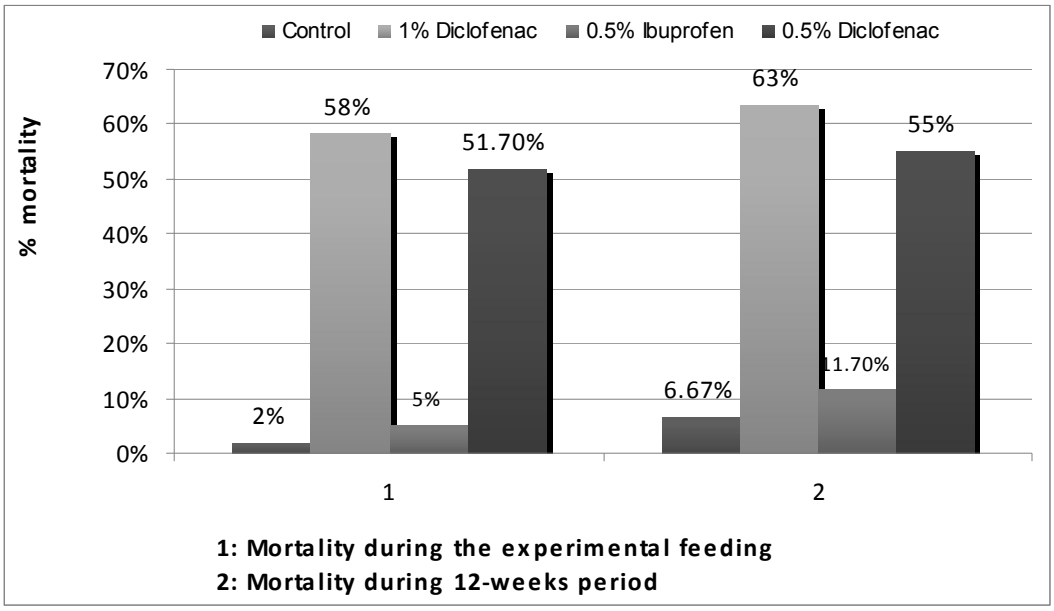

Figure 1. Mortality during the experimental feeding and during 12-weeks period.

Mortality was high in both diclofenac fed groups with percentage mortality of (58%) in the (1% diclofenac group) and (51.7%) in (the 0.5% diclofenac group) respectively during the experimental feeding compared to control and ibuprofen (Figure 1). This indicates a toxic potential of diclofenac toward fish. Investigations of (Schwaiget et al 2004) of diclofenac toxicity on rainbow trout revealed that the lowest observed effect concentration (LOEC) at which both renal lesions and alteration of gills occurs is 5 ugL^{-1}, with concentration related accumulation of the drug in all organ examined, and the highest amount detected in liver, followed by kidney, gills and muscle tissue. Gills alterations may interfere with normal respiratory functions and renal changes might lead to impaired osmo regulation. Furthermore prostaglandins are involved in many physiological functions eghomeostasis and nervous system function of which blocking could enhances the non-specific toxic effects. So far, there are no data available on the chronic toxicity of ibuprofen to fish except its toxicity on aquatic organism *Daphnia magna*, the mollusk *Planorbis carinatis* (Pounds et al 2008) which had been reported to be in mgL^{-1} range and the study carried on adult Japanese Medaka, *Oryzias latipes* showing that exposure to nominal concentrations of ibuprofen in the range of (1-100ugL^{-1}) for 6 weeks altered reproduction with no evident pathological damage in the gills, liver and kidneys of fish from the highest exposure group (Flippin et al 2007). Comparing different structures of both drugs, diclofenac is more water soluble with octanol-water partition coefficient log K_{ow} of 1.9 but in contrast ibuprofen is slight water soluble with log K_{ow} of 2.48. This further increase diclofenac toxic potential as more will be accumulated in water over 30 days feeding. In addition in vitro COX potency of diclofenac (IC50 0.06 um) is greater than ibuprofen (IC50 19 um), so diclofenac at equivalent concentration would be more potent than ibuprofen in vivo (Blain et al 2002).

Reproduction**:** The main goal of our study was to predict the effect of NSAIDS on sexual differentiation by using both diclofenac and ibuprofen during the crucial period of sexual differentiation where sufficient amount of estrogen is needed for proper ovarian development and the suppression of aromatase activity the rate limiting enzyme in aromatization of androgens to estrogens leads to sex reversal and the arise of male population (Kown et al 2001). 7% In the (control group), 36% in the (1% diclofenac group), 17% in the (0.5% ibuprofen group), and 22.2% in the (0.5% diclofenac group) respectively never produced egg during the entire experimental period (Figure 2).

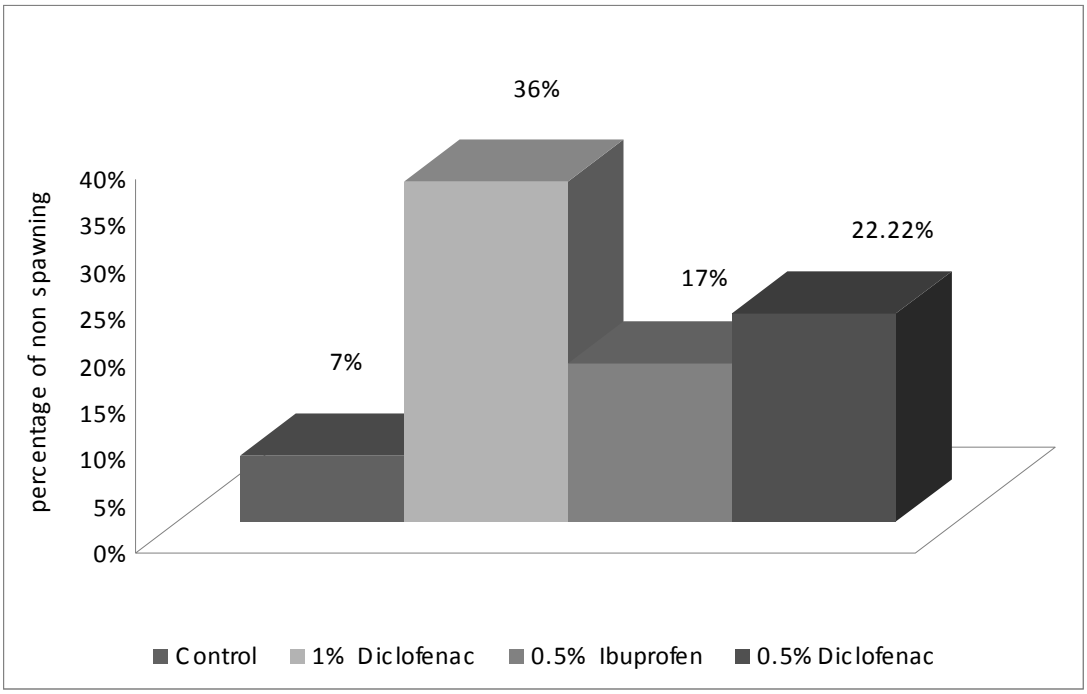

Figure 2. The percentage of non spawning fish during the experiment.

Macroscopically all the non-spawning fish in the experimental groups were females with apparently larger ovaries and full of eggs compared to control (Figure 3 and Figure 4).

Microscopically, histological sections of the ovaries showed that all groups had morphologically similar eggs (Figure 5).

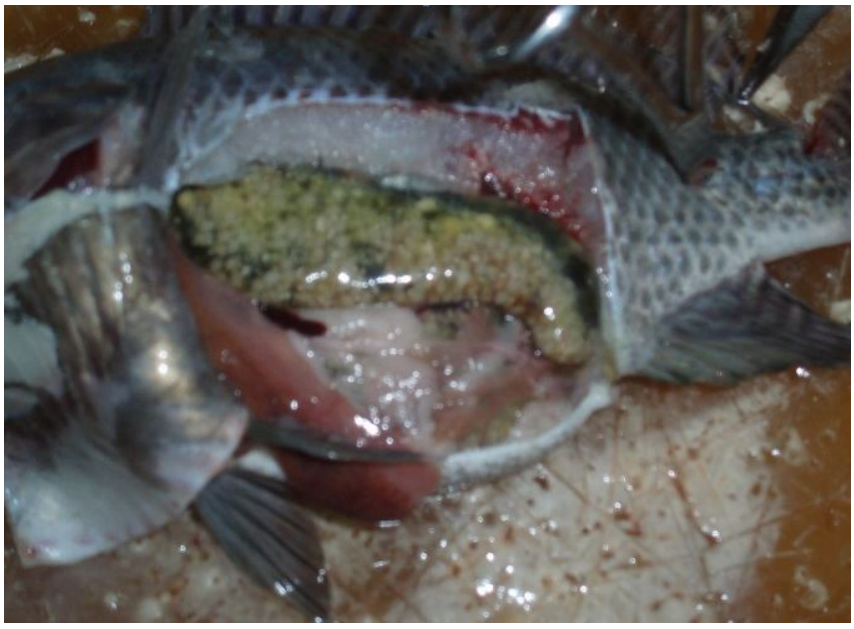

Figure 3. Ovaries filled with eggs in a fish dissected from (1% Diclofenac group).

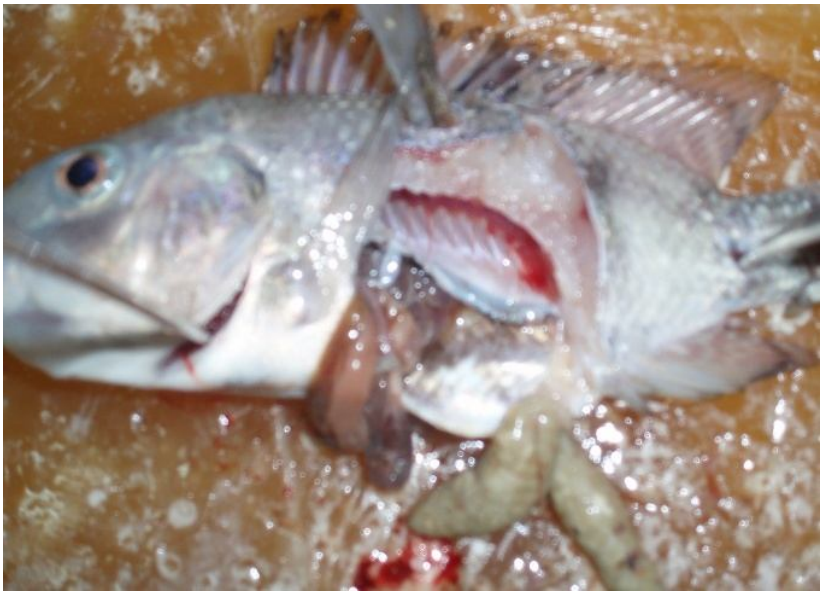

Figure 4. Ovaries in fish dissected from the (control group).

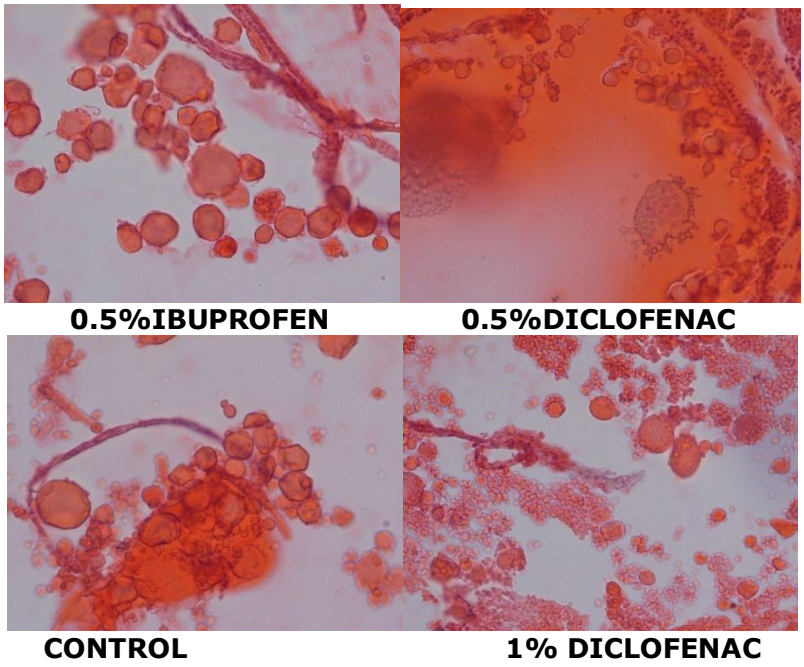

Figure 5. Histological sections of the ovaries from the different groups showing normal egg morphology.

Previous studies showed that Ibuprofen in the ranges of (1-100 ugL^{-1}) decreased the number of spawning events and increased the number of eggs per reproductive cycle in adult Japanese medaka (Flippin et al 2007). Cyclooxygenase inhibitors suppress aromatase expression and activity in breast cancer cells (Diaz-Cruz et al 2005) and since fish posses both COX-1 and COX-2 forms and both are widely expressed during development with both isoforms genetically and functionally homologous to their mammalian orthologs (Grosser et al 2002), we tested the hypothesis of possible cyclooxygenase inhibition during the crucial period that may modulate aromatase activity, thus altering sexual differentiation. Our observation of significant infertile female

population in the experimental groups following exposure at this early stage indicates that: NSAIDS could alter aromatase activity needed for proper sexual development and reproduction as mentioned earlier. We propose that alteration of aromatase activity at the transcriptional level by the COX-inhibitors decreases the rate of aromatization which alters E2 (estrogen) levels. Low (E2) release the feedback inhibition on gonadotropins producing high FSH levels that inhibits ovulation, since it is present in the blood of immature fish and levels increase during the vitellogenic phase but should decline towards follicular maturation and spawning (Kawauchi et al 1989).

Studies showed that many endocrine disruptive chemicals could alter aromatase cyp19 expression or activity at both transcriptional and post transcriptional levels and this is evident by the presence of multiple transcriptional regulatory elements which include cyclic adenosine monophosphate (cAMP) responsive elements, a steroidogentic factor 1/adrenal 4 binding protein site, an estrogen responsive elements (ERE), half-ERE'S, dioxin-responsive element and elements related to diverse other nuclear receptors and the cAMP responsive elements (CREs) were predicted in the 5-flanking regions of both cyp19a and cyp19b genes in tleosts (Cheshenko et al 2008). cAMP responsive element binding protein (CREB) regulates the transcription upon activation by phosphorylation with protein kinase A upon elevation of intracellularc AMP levels. Around 300 different stimuli can provoke CREB phosohorylation (Johannessen et al 2004). The use of NSADS like diclofenac and ibuprofen inhibit COX-1 and COX-2 irreversibly, thus inhibiting the production of prostaglandins (PGS) and lowers intracellular, CAMP, which could result in decreased aromatase expression via affecting promoter through CAMP responsive elements (CRE) at the transcriptional level. In addition, prostaglandin metabolites have also been shown to activate transcriptional targets directly in mammals, by binding to peroxisome proliferative-activated receptors (PPARs) which are members of orphan nuclear superfamily, that constitutively bind to the promoter elements of their target genes (Forman et al 1997). PPARs super family was identified in tilapia (Chang et al 2005).

Other possible postulated mechanisms could be through free ArA (arachidonic acid) generated by blocking the cyclooxygenase pathway (COX-1 and COX-2), which can modulate mRNA cyp19 expression by altering the binding of estrogen to its estrogen receptor elements (ERE) thus down regulating cyp19 expression, indeed estrogen will not be synthesized in sufficient amount, or it can modulate cyp19 activity at the post transcriptional levels by competing with the substrate for binding to active site of the enzyme. Endogenous long chain fatty acids are known to be regulators of cell signaling pathways and to affect either positively or negatively the binding of steroid hormones to their specific plasma proteins and their specific intracellular receptors and have also shown to co-regulate glucocorticoid-dependent gene expression (Sumida 1995). PPARs family members exhibit also a strong binding affinity for both saturated and unsaturated fatty acids which may indicate that the increase in free arachidonic (ArA) from cyclooxygenase inhibition could have an inhibitory effect on aromatase though binding to PPARs receptors (Mu et al 2001). On the other hand the observed non-spawning fish (7%) in the control group (Figure 2) could be accounted to captive conditions that may lead to dysfunction of one or more sites along the brain-pituitary-gonadal (BPG) axis (Zohar & Mylonas 2001).

Conclusions. This is the first time to our knowledge that NSADS (diclofenac and ibuprofen) are used as aromatase inhibitors to study sex reversal among Nile tilapia in vivo. The results of this study showed that the use of NSADS in fry fish during the crucial period may modulate aromatase activity and affects reproduction later in Nile tilapia, a fact that could be mediated by aromatase enzyme modulation. Furthermore, this is the first time we test the effects of these agents on fry teleosts during the crucial period of sexual development which points to possible alterations in reproduction following chronic exposure to these drugs at an early stage in contaminated surface water. As a consequence, we recommend to use COX-inhibitors in closed ponds where there is no contact with surface water in order to protect aquatic environment.

On the other hand, hormonal modulated products may have unfavorable effects on the human health, a fact that increases the need for a more environmentally friendly substitute. Based on this fact, it is obvious that we should select the more environmentally friendly substitute with the less side effects. Since results showed non-significant mortality with ibuprofen, in addition to its chemical properties that renders it less toxic to fish and does not accumulates in tissues. Ibuprofen could be considered a safer agent to fish.

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