



EFFECT OF 17 α -METHYLTESTOSTERONE RESIDUES ON RATS' LIVER

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ABSTRACT:

Introduction In current days most of Tilapia Nilotica (*Oreochromis Nilotica*) in Egyptian market comes from Monosex farming by using 17 α -Methyltestosterone (MT) sometimes misuse of this hormone cause harmful effect on a human who consumed fish containing MT hormone residues as it cause problems in puberty for girls and boys, liver tumors, carcinoma and increases embryo mortality.

Aim To assess the presence of MT residues in Tilapia Nilotica and to examine the effect of the exposure to this hormone on rat liver.

Subject and methods Tilapia Nilotica samples were collected from Kafr EL-sheikh monosex farms and MT residues were measured by ELISA then an experiment was done on 40 male rats divided to 4 groups 10 in each one control group, F group which take with its ration mixed fish flesh about two kilograms contain MT residues, A group take oral doses of MT 20mg/ kg body weight for 30 days and A group which take 80 mg /kg body weight for 30 days then blood samples collected for biochemical parameters (Alt, Ast, albumin, globulin, total protein, A/G ratio, total cholesterol, and HDL-c) and liver collected for monitoring CYP P450 E1 gene expression and TNF- α gene expression and histopathological examination of liver tissue.

Results We noticed the presence of hormone residues in Tilapia Nilotica Flesh by ELISA as the mean of hormone concentration was about 2.55 ppb which was above permissible limit and oral dosing of MT. The exposure of rats to MT leads to significant induction of gene expression of CYP P 450 E1 gene as the fold change on control group was one on both genes while in F group, A group, and B group (2.07 ± 0.13 , 6.96 ± 0.19 and 13.2 ± 0.41) respectively and also TNF- α gene as F group, A group and B group was (2.85 ± 0.05 , 8.84 ± 0.19 , and 15.4 ± 0.32) respectively ($P \leq 0.05$). Liver functions tests reveal increased ALT and AST values in group B. Histopathology reveals hydropic changes in the B group. MT caused hypocholesterolemic effect as there is a significant decrease in total cholesterol value from 70.5 ± 2.63 mg/dl in the control group to 46.2 ± 2.32 mg/dl in the B group and HDL-c also, decreased significantly from 46.2 ± 2.32 mg/dl in the control group to 40.0 ± 2.65 mg/dl ($P \leq 0.05$).

Conclusion MT is a hepatotoxic substance as exposure to MT caused induction of some inflammatory mediators as CYT. P450 E1 and TNF- α genes. MT may be needs a long term of exposure to show hepatic dysfunction, necrosis and carcinoma as high oral dose (80mg/kg) for one month causes only hydropic changes. MT decrease total cholesterol in a bad way decreasing lipoprotein HDL which may lead to atherosclerosis.

Keywords: 17 α Methyltestosterone (MT), Cytochrome P450E1, Tumor Necrosis Factor α (TNF- α), and *Oreochromis Nilotica*.

Oreochromis Nilotica has bound favorable characteristics, that create its cultivation most tolerant to adverse environmental conditions. Also, it has the ability to survive at low dissolved oxygen with relatively fast growth and efficient food conversion.⁽¹⁾ Because of their high macromolecule content, large size, ascension (6–7 months to grow to reap size) and taste property, genus *Tilapia* is within the focus of major cultivation. Nile tilapia is a native fish species of Egypt that has become popular worldwide, mainly as a valuable fish, easy to breed and grows in a variety of aquaculture systems.⁽²⁾

Currently, aquaculture is a promising global agricultural industry.⁽³⁾ Nile tilapia aquaculture production around the world resulted in 2,537,492 tons in 2010 and 3,425,121 tons in 2013. Aquaculture production of Nile tilapia in Egypt represented about 81%, 88% and 86% of tilapia yields in 2010, 2012 and 2013, respectively.⁽⁴⁾

There is a need for monosex culturing, especially of all-male fish. All-male monosex culture is characterized by greater uniformity in growth, size and body weight, better flesh quality, more rapid growth and the lack of spawning.⁽⁵⁾ Androgens, such as 17 α -methyl testosterone (MT), and carp testis (natural androgen), are widely used in this respect⁽⁶⁾ as well as trenbolone or nonsteroidal aromatase inhibitors, such as fadrozole, which

efficiently produce a higher percentage of male fish. MT has been used for the production of monosex stock in several species, such as yellow catfish, spotted scat, medaka, Atlantic cod, and guppy (*Poecilia reticulata*). In Nile tilapia, masculinization processes begin when larvae are just finished the yolk sac absorption, at which time, they are fed a diet containing MT at doses of 40, 50, 60 or 70 mg MT/kg until 28 days of age. ⁽⁷⁾

TNF- α activates several intracellular pathways to regulate inflammation, cell death, and proliferation. In the liver, TNF- α is not only a mediator of hepatotoxicity but also contributes to the restoration of functional liver mass by driving hepatocyte proliferation and liver regeneration (8). It was known that hepatic expression of the proinflammatory cytokine tumor necrosis factor- α (TNF- α) occurs in many acute and chronic liver diseases, as well as following exposure to hepatotoxic chemicals, and is believed to help influence each the damage and repair processes that occur following these insults by control extra mediators. ⁽⁹⁾ Other hepatic harms associated with anabolic- androgen steroids abuse include liver alterations such as subcellular changes of hepatocytes, hepatocellular hyperplasia, and general hepatic damage determined by increased liver enzymes. ⁽¹⁰⁾ Also, gene expression of each CYP is influenced by a unique combination of mechanisms and factors including genetic polymorphisms, induction by xenobiotics, regulation by cytokines, hormones and during disease states, in addition to sex, and age. ⁽¹¹⁾ The use of MT is a critical issue and sometimes occur misuse of hormonal treatments in sex reversal of tilapia especially in the Egyptian private sector hatcheries, our research main objective is to evaluate food safety of *Tilapia Nilotica* which comes from monosex farms by measuring MT residues to detect its absence or presence within permissible limit in *Tilapia Nilotica* muscles.

SUBJECTS AND METHODS

A pilot test was done for detection presence or absence of 17- α Methyltestosterone (MT) residue in *Tilapia Nilotica* muscle. Fish samples were collected from Kafr El-Sheikh farms which producing Monosex *Tilapia Nilotica* (fish weight ranges from 100-350 gram). The samples were collected in polyethylene bags and transferred rapidly to the laboratory for detection of MT hormonal residues.

Kit used in this work as the following; kit from (R-Biopharm AG, Darmstadt, Germany), Kits for measuring Biochemical parameters from Cobas Chemicals, Roche Diagnostics, USA. Kits for gene expression: RNeasy Mini Kit (Qiagen GmbH, Germany), QuantiTect Reverse Transcription Kit (Qiagen), and QuantiTect SYBR Green PCR Master Mix (Qiagen Rotor-Gene Q).

Forty male Wistar Albino rats weighing 180-200 g were purchased from Animal breeding unit, Faculty of Agriculture Alexandria University; the animals were kept in metal cages under environmental-controlled conditions with optimum temperature, humidity, and dark/light cycle

and free access to rat chow and drinking water. The international ethical guidelines for the care and use of laboratory animals were performed to handle the animals and the experimental procedures were approved by the Experimental Animal Use and Ethics Committee at the Faculty of Veterinary Medicine, Alexandria University, Egypt. The rats were randomly assigned to four groups (10 rats each).

C group: control group.

F group: the group which take *Tilapia Nilotica* flesh that contains residues of Methyltestosterone previously estimated by ELISA about two kilograms fish mixed randomly with starter ration daily for 30 days,

A group: the group which take 20 mg/kg body weight oral dose of Methyltestosterone daily for 30 days and

B group: which take 80 mg/kg body weight oral dose of Methyltestosterone daily for 30 days.

After the end of experiment, blood samples were collected for measuring biochemical parameters and liver exposed to a molecular and histopathological investigation.

Detection of Methyltestosterone residues in *Tilapia Nilotica* Flesh by ELISA;

Extraction of muscle: Skin and scales were removed from the muscle of fish, then ten grams of the ground muscle were homogenized with 10 ml of 67mM PBS buffer by mixer for 5 minutes, two grams of homogenized sample were mixed with 5 ml of Tertiary Butyl Methyl Ether (TBME) in a centrifugal screw cap vial and shaken vigorously by a shaker for 30-60 minutes, the contents were centrifuged at 3000 rpm for 10 minutes, the supernatants were kept and the extraction with TBME was repeated, the supernatants were combined and evaporated then the dried extract was dissolved in 1 ml of 40% Methanol, the methanolic solution was diluted with 2 ml of 20 mM PBS- buffer and applied to a RIDA C18 column (Solid phase extraction column with C18 end-capped sorbent of an average particle size of 50 μ m Art No. R2002, flow rate: 1 drop/second.

The test procedures of ELISA were done according to the chart enclosed in the kits of RIDAR and RIDS screen. (R-Biopharm AG, Darmstadt, Germany). Methyltestosterone standard solutions used for the calibration curve levels of 0, 50, 150, 450, 1350 and 4050 ng/L (ppt) and were all included in the ELISA test kit pamphlet (R-Biopharm AG, Darmstadt, Germany). ⁽¹²⁾

RNA Extraction and q RT-PCR; About 100 mg liver tissues were rinsed in sterilized phosphate buffer saline and homogenized in liquid nitrogen using Teflon and pestle homogenizer then the homogenates were stored at -80°C till RNA isolation. Total RNA was isolated using the RNeasy Mini Kit (Qiagen GmbH, Germany) according to the manufacturer instructions. cDNA was synthesized from the purified RNA using the QuantiTect

Reverse Transcription Kit (Qiagen). The qRT-PCR for the target genes were performed using QuantiTect SYBR Green PCR Master Mix (Qiagen Rotor-Gene Q). The primer sequences of all target and reference genes and the PCR conditions were recorded in Table 1. The cycling conditions for SYBR green real-time PCR according to Quantitect SYBR green PCR kit Table 2. The fold change of mRNA expression was calculated after recording the Ct values for reference and target genes using the $2^{-\Delta\Delta Ct}$ method. ⁽¹³⁾

Serum samples were used to assess liver function tests; ALT, ⁽¹⁴⁾ AST, ⁽¹⁴⁾ total protein, ⁽¹⁵⁾ albumin, ⁽¹⁶⁾ globulin, ⁽¹⁷⁾ and A/G ratio ⁽¹⁷⁾ in addition to total cholesterol, ⁽¹⁸⁾ and HDL-c ⁽¹⁹⁾ using kit Cobas Chemicals, Roche Diagnostics, USA.

Histopathological Examination; at the end of experiment the liver of each rat was fixed in 10% formalin solution. The samples were cleared in xylene and immersed in paraffin. The paraffin blocks were sectioned at 5 mm thickness and mounted on clean glass slides. The ordinary Hematoxylin and Eosin stains were used and then read under an optical microscope. ⁽²⁰⁾

STATISTICAL ANALYSIS

All variables were presented as mean \pm standard error of the mean. A probability value of 0.05 or less was considered statistically significant. All analyses were performed using the SPSS statistical software package (version 22.0 SPSS Inc., Chicago, USA). First, tests of normality were performed on raw data. For normally distributed variables, we used one-way analysis of variance (ANOVA) for the effect of group followed by Waller-Duncan's post-hoc. Non-normally distributed variables were compared with the non-parametric Kruskal-Wallis test with Mann-Whitney U-test for pairwise differences.

RESULTS

A pilot test was done on ten Nile Tilapia randomly obtained from Kafr El-Sheikh monosex farms to assess the presence of MT residues in Tilapia Nilotca flesh by using ELISA. Result showed that mean value of MT residues in Tilapia Nilotca flesh was 2.55 ppb ($\mu\text{g}/\text{kg}$) and the standard deviation was 0.86.

The hepatic CYT.P450E1 and TNF- α gene expression are presented in Table 3 and Figure 1 which shows a dose dependant increase in fold change of expression of CYT. P450E1 and TNF- α genes. As the exposure of rats to MT leads to significant induction of gene expression of CYP

P 450 E1 gene as the fold change on control group was one on both genes while in F group, A group, and B group (2.07 ± 0.13 , 6.96 ± 0.19 and 13.2 ± 0.41) respectively and also TNF- α gene as F group, A group and B group was (2.85 ± 0.05 , 8.84 ± 0.19 , and 15.4 ± 0.32) respectively ($P \leq 0.05$).

Liver function tests include ALT, AST, albumin, total protein, globulin, and A/G ratio in the rat exposed to different oral doses for 30 days, changes in liver function tests as shown in Table 4. ALT did not change in group F and A but increased significantly in group B when compared to control group 35.7 ± 1.61 U/l, 37.8 ± 2.79 U/l, 46.3 ± 4.57 U/l and 37.0 ± 1.81 U/l respectively ($P \leq 0.05$). AST also did not change in group F and A but increased significantly in group B when compared to control group 131 ± 8.87 U/l, 158 ± 11.7 U/l, 194 ± 11.5 U/l and 143 ± 8.21 U/l respectively ($P \leq 0.05$). Total protein did not change significantly in F, A and B groups compared to control group 6.13 ± 0.32 , 5.92 ± 0.39 g/dl, 6.58 ± 0.22 g/dl, and 6.73 ± 0.10 g/dl respectively ($P \leq 0.05$). Albumin did not change significantly in F, A and B groups compared to control group 3.92 ± 0.25 g/dl, 3.75 ± 0.30 g/dl, 4.22 ± 0.20 g/dl, and 4.20 ± 0.04 g/dl respectively ($P \leq 0.05$). Globulin did not change significantly in F, A and B groups compared to control group 2.22 ± 0.12 g/dl, 2.17 ± 0.13 g/dl, 2.37 ± 0.07 g/dl, and 2.53 ± 0.07 g/dl respectively ($P \leq 0.05$). Also A/G ratio did not change significantly in F, A and B groups compared to control group 1.78 ± 0.11 , 1.73 ± 0.11 , 1.79 ± 0.09 , and 1.67 ± 0.04 respectively ($P \leq 0.05$).

Effect of MT on total cholesterol, and high density lipoproteins; rats which exposed to high dose 80 mg/kg body weight for 30 days (group B) shows a lower cholesterol level but not in a healthy way as HDL-c was significantly decreased. As in Table 5. Total Cholesterol decreased significantly in group B as in the control group was 70.5 ± 2.63 mg/dl and in group B 46.2 ± 2.32 mg/dl while F and A groups did not change significantly as their values were 70.8 ± 2.27 and 63.5 ± 4.81 respectively. Also HDL-c was in the control group 51.5 ± 2.64 mg/dl, group F increased slightly becomes 57.7 ± 3.21 mg/dl and in group B decreased significantly become 40.0 ± 2.65 mg/dl while in group A decreased but not as significant decrease as it was 49.0 ± 5.06 mg/dl ($P \leq 0.05$).

Histopathological examination of liver sections of control rats reveals normal hepatic architecture formed of lobules of hepatocytes separated by sinusoidal blood vessels and the portal tract contains inflammatory cells, while in F group reveals hepatocytes separated by sinusoidal blood vessels and the hepatocytes showing mild nuclear enlargement. In A group the hepatocytes showing mild to moderate nuclear enlargement, hyperchromatism, and coarse chromatin. Finally, in B group the hepatocytes showing hydropic changes also, it shows moderate nuclear enlargement and coarse chromatin.

Table1. Oligonucleotide primers sequences used in SYBR Green real time PCR
Source: Metabion (Germany).

Gene	Primer sequence (5'-3')	Reference
Rat β -actin*	TCCTCCTGAGCGCAAGTACTCT	(21)
	GCTCAGTAACAGTCCGCCTAGAA	
CY450P2E1	CTCCTCGTCATATCCATCTG	(22)
	GCAGCCAATCAGAAATGTGG	
TNF- α	CACCAGCTCTGAACAGATCATGA	(23)
	TCAGCCCATCTTCTTCCAGATGGT	

*Housekeeping gene.

Table 2. Cycling conditions for SYBR green real time PCR according to Quantitect SYBR green PCR kit:

Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)		Final denaturation
		Secondary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	Annealing	
50°C 30 min.	94°C 5 min.	94°C 15 sec.	60°C 30 sec.	72°C 30 sec.	94°C 1 min.	60°C 1 min.	94°C 1 min.

Table 3, Figure 1.A and Figure 1.B : shows significant increase in fold change of gene expression of Cyt.P450E1 and TNF- α genes according to oral dose of MT.

Group	TNF- α	Cyt. P. 450 E1
C	1.00 \pm 0.00 ^d	1.00 \pm 0.00 ^d
F	2.07 \pm 0.13 ^c	2.85 \pm 0.05 ^c
A	6.96 \pm 0.19 ^b	8.84 \pm 0.19 ^b
B	13.2 \pm 0.41 ^a	15.4 \pm 0.32 ^a

Groups: C, Control; F, group which eat Tilapia containing MT residues; A, group which take dose 20mg/kg MT; B, group which take dose 80mg/kg.

Values are means \pm standard errors.

Means without a common superscript in a column differ significantly ($P \leq 0.05$).

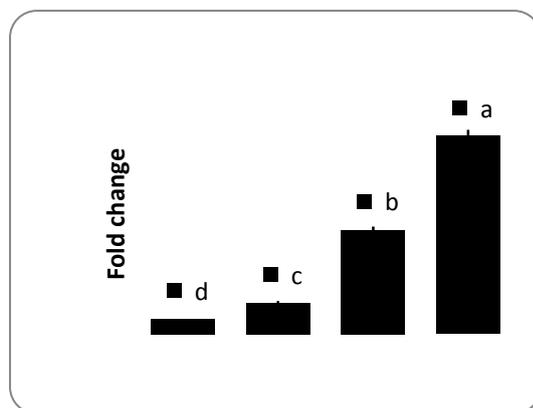


Fig.1 (A)

Fig. 1 Effect of MT on TNF- α and Cyt.P450 E1 genes:

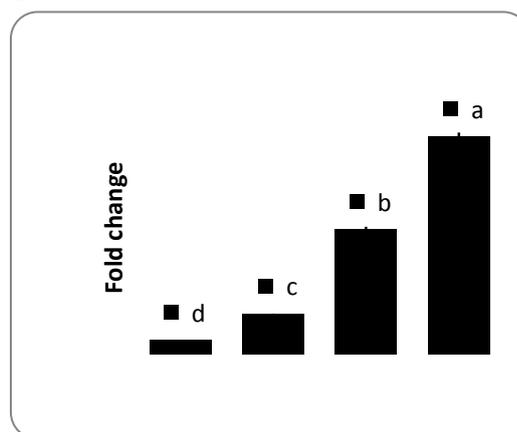


Fig.1 (B)

Fig. 1 Effect of MT on TNF- α and Cyt.P450 E1 genes:

Fig.1 (A) shows the effect of MT on TNF- α genes, **Fig. 1 (B)** shows the effect of MT on Cyt.P450 E1

Groups: C, Control; F, group which eat Tilapia containing MT residues; A, group which take dose 20mg/kg MT; B, group which take dose 80 mg/kg.

Values are means \pm standard errors.

Means without a common superscript in a column differ significantly ($P \leq 0.05$).

Table 4. Rats' serum liver function tests:

Groups	ALT(U/l)	AST(U/l)	Total protein(g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G Ratio
C group	37.0±1.81 ^b	143±8.21 ^b	6.73 ± 0.10 ^a	4.20±0.04 ^a	2.53±0.07 ^a	1.67 ± 0.04 ^a
F group	35.7±1.61 ^b	131±8.87 ^b	6.13 ± 0.32 ^a	3.92±0.25 ^a	2.22±0.12 ^{ab}	1.78 ± 0.11 ^a
A group	37.8±2.79 ^{ab}	158±11.7 ^b	5.92 ± 0.39 ^a	3.75±0.30 ^a	2.17 ± 0.13 ^{ab}	1.73 ± 0.11 ^a
B group	46.3±4.57 ^a	194±11.5 ^a	6.58 ± 0.22 ^a	4.22±0.20 ^a	2.37±0.07 ^{ab}	1.79 ± 0.09 ^a

Groups: C, Control; F, group which eat Tilapia containing MT residues; A, group which take dose 20mg/kg MT; B, group which take dose 80mg/kg

Values are means ± standard errors.

Means without a common superscript in a column differ significantly ($P \leq 0.05$).

Table 5. Serum total cholesterol and serum high density lipoprotein cholesterol:

Group	Total cholesterol (mg/dl)	HDL-C (mg/dl)
C	70.5 ± 2.63 ^a	51.5 ± 2.64 ^a
F	70.8 ± 2.27 ^a	57.7 ± 3.21 ^a
A	63.5 ± 4.81 ^a	49.0 ± 5.06 ^{ab}
B	46.2 ± 2.32 ^b	40.0 ± 2.65 ^b

Groups: C, Control; F, group which eat Tilapia containing MT residues; A, group which take dose 20mg/kg MT; B, group which take dose 80mg/kg.

Values are means ± standard errors.

Means without a common superscript in a column differ significantly ($P \leq 0.05$).

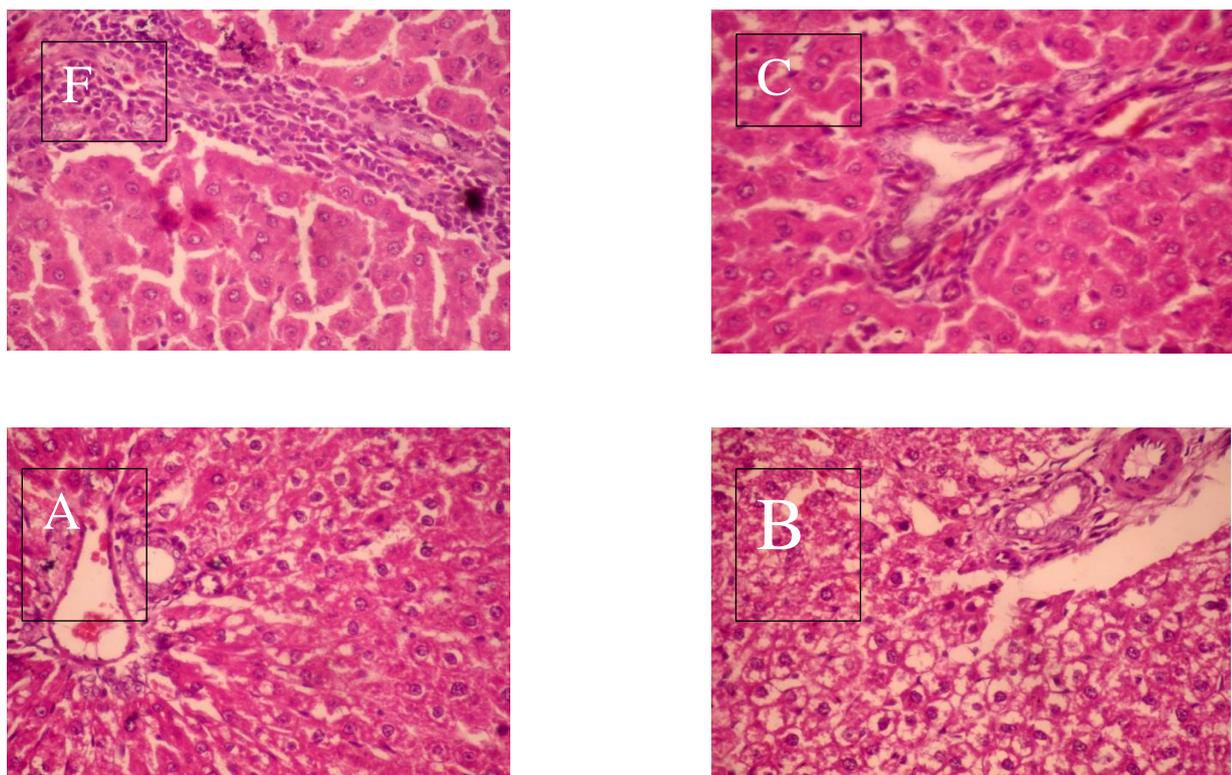
**Fig. 2 H and E stained section of hepatic tissue**

Fig. C (C group) reveals normal hepatic architecture formed of lobules of hepatocytes separated by sinusoidal blood vessels and the portal tract contains inflammatory cells. Inflammatory Cells, Hepatocyte, and blood sinusoids. **Fig. F** (F group) reveals hepatocytes separated by sinusoidal blood vessels and the hepatocytes showing mild nuclear enlargement. As shown by a blue arrow. **Fig. A** (A group) reveals hepatocytes separated by sinusoidal blood vessels and the hepatocytes showing mild to moderate nuclear enlargement, hyperchromatism, and a black arrow shows coarse chromatin. **Fig. B** (B group) reveals hepatocytes separated by sinusoidal blood vessels and the hepatocytes showing moderate nuclear enlargement, black arrow shows coarse chromatin, hyperchromatism, and the star shows hydropic changes. (H and E stain X40).

Discussion

The ELISA method is the most applicable in official control laboratories as a rapid and accurate screening method for the determination of Methyltestosterone in fish⁽²⁴⁾. The study clearly indicated the presence of MT in fish muscle as the mean value of Methyltestosterone residues in examined fish flesh is about 2.55 ± 0.86 ppb in fish muscle and this exceeds the permissible limit according to European Food Safety Authority which is 0.64 ppb in muscle⁽²⁵⁾. Also exceed the permissible limit of Codex Alimentarius which is 2 ppb in muscle⁽²⁶⁾. The detection of hormonal residues in Tilapia mainly may be attributed to widely use of synthetic androgens as Methyltestosterone in fish production in Egypt for its anabolic and androgenic actions⁽²⁷⁾.

In examined rat liver we found a significant increase in gene expression of TNF- α as a result of exposure to MT hormone. Hepatic expression of the proinflammatory cytokine TNF- α occurs in many acute and chronic liver diseases, as well as following exposure to hepatotoxic chemicals, and is believed to help influence each the damage and repair processes that occur following these insults by control extra mediators⁽⁹⁾. TNF- α is considered one of the main mediators of initial liver fibrosis and liver cirrhosis development even with normal liver function tests⁽²⁸⁾.

The results indicated a significant dose dependant increase in cytochrome P450 E1 as a result of exposure to MT. Cytochrome P450 mono-oxygenases (CYP) play an essential role in steroid metabolism. Cytochromes P450 is a major source of variability in drug pharmacokinetics and response. Of 57 putatively functional human CYPs only about a dozen enzymes, belonging to the CYP1, 2, and 3 families, are responsible for the biotransformation of most foreign substances including 70–80% of all drugs in clinical use. Expression of each CYP is influenced by a unique combination of mechanisms and factors including genetic polymorphisms, induction by xenobiotic, regulation by cytokines, hormones and during disease states, also sex, and age⁽¹¹⁾.

Gross examination of the liver during rat dissection showed hepatomegaly especially in group A and group B. Liver function tests which include ALT, AST, albumin, total protein, globulin and A/G ratio rat exposed to different oral doses for 30 days this period does not show significant changes in liver function tests as shown in Table 3. except in group B ALT increased significantly, also AST value increased significantly especially in B group. These results agree with data demonstrated that 17 α - Methyltestosterone has the most hepatotoxic effect than other forms of synthetic anabolic steroids⁽²⁹⁾. It was known that muscle has more AST and ALT when compared with that in the liver because of a larger tissue mass⁽³⁰⁾. Consequently, transaminase levels would be elevated owing to various types of muscular disorders or injury (e.g., heart attack, surgery, and vigorous exercise), hemolysis, and small bowel ischemia and in our study, this may be due to higher body weight⁽³⁰⁾.

Histopathological examination of liver tissue reveals nuclear enlargement, hyperchromatism, and coarse chromatin and finally hydropic changes in hepatocytes in group B which received 80 mg/kg MT oral doses for 30 days. In our study, hepatic necrosis did not occur just hydropic changes in hepatocytes and this might be contributed to oral dosing need long term exposure to cause hepatic necrosis than other routes of exposure to MT as subcutaneous injection⁽³¹⁾.

CONCLUSION

From this study which revealed presence of MT residues in Tilapia flesh as a result of misuse of this hormone in monosex farming and we recommend application of strict regulation by Egyptian authorities mainly on private farms with training and education to worker in order to ensure adequate use of MT to give good quality Tilapia free from MT residues and safe to consumer.

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