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Cytogenetic Evaluation of Hormonally Sex-Reversed *Oreochromis niloticus* Following Hormone Cessation in the Absence and Presence of Heat Stress

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Abstract: Production of all male populations (monosex) of tilapia through treatment of fry with 17α-methyl testosterone (MT) impregnated food has become common. The aim of this study is cytogenetic evaluation of 17á-methyl testosterone sex-reversed tilapia following cessation of hormone treatment in the presence and absence of heat stress by detection of mitotic index and nuclear anomalies (micronuclei and chromosomal aberrations). Two different ages were used, MT treated fish at 1 and 3.5 months after cessation of hormonal treatment and the corresponding age of non-MT treated tilapia fish (*Oreochromis niloticus*) as a control. The fish were obtained from a farm use agricultural plant discharge as a source of water. The heat stress temperature used was 38°C. The results showed that, there is no significant difference in nuclear anomalies between MT and non-MT treated fish at normal or heat stress conditions. Moreover, there is no significant difference in mitotic activity in all groups and it was not altered by heat stress, which indicate the same growth rate in MT and non-MT treated fish and their resistance to the effect of heat stress. Furthermore, this study demonstrated that heat stress is genotoxic in MT and non-MT treated fish indicated by increased number of nuclear anomalies especially in younger age fish. The results indicated that MT treatment has no clastogenic effects and the heat stress is genotoxic to MT and non MT treated fish.

Key words: 17α-Methyl Testosterone · Heat Stress · Genotoxicity · *Oreochromis niloticus* · Sewage Plant Discharge

INTRODUCTION

Production of all male population through administration of 17α-methyl testosterone (MT) is considered to be the most effective and economically feasible method for obtaining all male tilapia population [1]. A 98.3 % male population was obtained when fish fry fed a MT (60 mg MT kg^{-1}) treated feed for 28 days [2]. Many authors have investigated the effect of oral administration of MT on sex chromosome, oral administration of MT generally resulted in sex reversal and the hormone is readily metabolized in several tilapiine species [3, 4]. However, high doses may results in a reduction of gonadal growth, gonadal intersexuality and feminization [5, 6] or abnormal gonadal development such as fibrosis and necrosis resulting in sterility [7, 8]. There are also some reports on alterations in spermatogenesis such as inhibition of spermiation, low fertility and poor reproductive performance in several teleostean species after treatment with MT [9].

On the other hand reports for the genotoxic effect of MT administration on somatic cell of fish are scarce. However, Timchenko *et al.* and Tsutsui *et al.* [10, 11] found that Testosterone propionate showed genotoxic effect in vivo and in vitro. Furthermore, high doses of this compound induced chromosomal stickiness, breaks and varying chromosomal irregularities in *Notopterus notopterus* fish [12].

Practical fish health management is based on stress management. Stress is an abnormal physiological condition of fish that results when the fish's collective adaptive responses to environmental factors are extended to its limit of tolerance. When fish are continuously exposed to stress, their immune system

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becomes weakened. Consequently, their ability to fight disease is reduced and they then succumb to infections and fall sick. In severe or prolonged cases, this may lead to death [13-15].

Exposure of cells or organism to thermal stress is known to result in alterations in the integrity of the nucleolus besides adversely affecting the structure and function of centromere [16, 17]. Delay in cell cycle progression, inability of the cell cycle progression and inability of the cells to inter mitosis has been observed [18]. The genotoxic effect on heat shock on gold fish has been detected [19].

Egypt has hot summer, with temperature range from 35-42°C and many fish farms use agriculture sewage discharge with its high contents of different pollutants as heavy metals and pesticides [20]. Furthermore, Methyl testosterone is susceptible to breakdown when exposed to light or high temperature [21].

The aim of this study is the cytogenetic evaluation of sex-reversed Nile tilapia following hormone treatment cessation in the absence and presence of heat stress with special reference to mitotic index and nuclear anomalies.

MATERIALS AND METHODS

Experimental Animals: Fish were obtained from private fish farm located in Edko, Behera Governorate, Egypt. The water supply of this farm is sewage plant discharge. Nile tilapia (*Oreochromis niloticus*) fish and 17α-methyl testosterone treated Tilapia nilotica (MT treated) were used. MT treated fish were obtained by feeding the *O. niloticus* fry MT treated feed for 28 days (60 mg MT kg^{-1}), the average weight of fry was 1.9 gm at the end of the treatment period.

Experimental Design

Two Different Age Groups Were Used:

Group 1: One month after cessation of hormonal treatment (MT treated group) and the corresponding age from non-MT treated *Oreochromas niloticus* Their weights ranged from 3-6 gm.

Group 2: 3.5 months after cessation of hormonal treatment (MT treated group) and the corresponding ages from non-MT treated *Oreochromas niloticus*. Their weights ranged from 9-13 gm.

Fish were maintained in glass aquaria (85x40x 65cm $aquarium^{-1}$). Dechlorinanitaed tap water were used, the volume of the water in the aquarium about one third of its volume, they were aerated continuously and water changed once in two days. Fish were acclimatized at ambient temperature (25°C) for 7 days before the experiment.

Protocol for Heat Stress: Heat stress experiment was done according to Anitha *et al.* [19]. Six fish from each group (MT treated, non MT treated groups at 1 and 3.5 months) were obtained for heat stress experiment and maintained in small glass jars. The heat stress temperature was 38°C. When the temperature of the water reached the desired level the fish were directly placed in the glass jars, they were left in the exposure temperature for 3 hrs and then transferred to control temperature (25°C). Six fish from the corresponding groups were used as a control without heat treatment.

Micronucleus Test: Micronucleus assay was carried out as described by Hooftman and Raat [22]. Twenty-four hours after heat stress peripheral blood was drawn with heparinized syringe from the caudal peduncle. Smears were made on clean glass slides and fixed in methanol for 15 min. Following fixation, the cells were treated in 1 N HCl for 20 min at 60°C and washed in cold 1 N HCl followed by distilled water. The slides were then stained in 8% Giemsa Gurr and polychromatic erythrocytes were observed under x100. Five hundred polychromatic erythrocytes were scored from each fish and six fish were tested for each treatment.

Chromosomal Analysis: Chromosomal analysis was carried out according to the procedure of [23]. Twenty-one hours after heat shock, the fish were injected with colchicines at a concentration of 25 mg per gm body weight. Three hours later the fish were sacrificed and gill filament tissue were obtained and minced with sharp scissor in 0.075 M KCl as a hypotonic solution at 37°C for 25 min. The cells were centrifuged for 5 min at 1000 rpm and then fixed in methyl acetic acid 3:1, then centrifuged at 1000 rpm for 5 min. The supernatant was discarded, the pellets resuspended in 5 ml of fixative and centrifugation repeated. This procedure was repeated twice. The cells spread into clean slides. The slide were air dried stained with 4% Gur Giemsa and 50 well spread metaphases per fish were selected for analysis of chromosomal aberrations including fragment, deletion, ring chromosome and end-to end association. The mitotic indices were calculated from 1000 cells per fish.

Statistical Analysis: All data were statistically analyzed by one-way analysis of variance (ANOVA). Multiple comparisons were performed by Duncan's multiple range test. All values reported as means±S.E. For all experimental data, the significance level was set at $P \leq 0.05$, when appropriate.

RESULTS

Micronucleus Assay: All groups of fish showed increased number of micronucleated erythrocytes with variable degrees (Fig. 1, Table 1). No significant difference in the number of micronuclei in MT and non-MT treated fish in both age groups under normal and heat stress conditions. Also, there is no age difference in the number of micronucleated erythrocytes in fish under normal condition. Heat stress induced significant increase in the number of micronuclei in MT and non-MT treated fish at both age groups. However, Micronucleated erythrocytes increased significantly in the younger age group 1 (MT and non-MT) than the older age group 2 (Table 1).

Mitotic Index: All fish at all groups showed no significant difference in mitotic index. No difference between MT and non-MT treated fish at both age groups. Heat stress also showed no effect on mitotic activity on both fish and both ages (Fig. 1A; Table 1)

Chromosomal Aberrations: All groups showed increased number of aberrant cells and some types of structural chromosomal aberrations, which include fragment, deletion, ring chromosome, end-to-end association (Fig. 1.B, C and D; Table 2). Few polyploidy cells were observed in 3 fish, which is not included in the tables because of its difficult statistical analysis. There is no significant difference between MT and non-MT treated fish in the number of aberrant cells or in the different types of chromosomal aberrations at either age groups. Also there is no age difference in nuclear anomalies between the two groups under normal condition.

Heat stress showed genotoxic effects indicated by a significant increase in the number of aberrant cells and different types of structural chromosomal aberration with variable degrees between two age groups. No difference in the number of aberrant cells or in the different types of structural chromosomal aberrations between MT and non-MT treated fish at both age groups under heat stress condition.

Table 1: Number of micronuclei and mitotic index in MT and non-MT treated fish at 1 and 3.5 months in the absence and presence of heat stress

Groups	Micronuclus* number	Mitotic index**
1 month***		
T	23.0±4.9^{c}	45.3±6.0
MT	34.3±4.8^{c}	42.5±3.9
T-MT	78.3±5.0^{a}	35.8±3.9
MT-HS	89.5±8.8^{a}	41.0±4.0
3 month***		
T	23.0±3.8^{c}	32.8±2.8
MT	33.3±2.7^{c}	37.3±2.4
T-MT	59.8±8.2^{b}	37.8±6.8
MT-HS	62.3±10.0^{b}	34.3±3.0

$^{a-c}$Values with different letters at the same column are significantly differed $P<0.05$

Each value represents the mean ± SE of six animals.

*Micronucleus incidence in 500 polychromatic erythrocyte.

** No. of dividing cells in 1000 cells.

*** 1, 3.5 months after cessation of hormonal treatment

MT= Methyltestosterone treated fish T = *Oreochromis niloticus* (non-MT treated),

T-HS = Heat stressed *Oreochromis niloticus*, MT-HS = Heat stressed MT treated fish

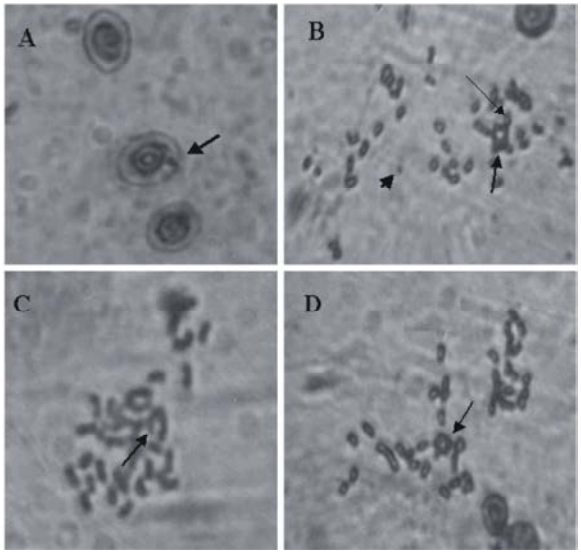

Fig. 1: Photomicrograph shows (A) micronucleated erythrocyte, (B) Fragment (arrow head) and end-to-end association (arrow), (C) Deletion, (D), Ring chromosome as represented lesions in fish under study

In younger age group, heat stress induced an increase in the number of deletion and ring chromosome. In older age group, there is an increase in the number of aberrant cells. Heat stress significantly increased chromosomal stickiness at both age groups indicated by increased number of end-to end association.

Table 2: Number of aberrant cells and different kinds of chromosomal aberrations reported in MT and non-MT treated fish at 1 and 3.5 months in the absence and presence of heat stress

Groups	No of Aberrant cells	Frag	Del.	Ring chromosome	End-to end association
1 month***					
T	22.0±1.3[b]	25.6±0.4[a]	2.2±1.1[b]	0.8±0.5[b]	3.6±1.2[b]
MT	22.6±1.1[b]	32.6±1.2[a]	1.0±0.5[b]	1.2±0.6[b]	6.6±2.3[b]
T-MT	26.8±3.3[a,b]	18.4±1.5[b]	5.2±0.5[a]	4.8±1.0[a]	11.6±3.5[a]
MT-HS	20.6±1.0[b]	19.2±2.6[b]	4.8±0.7[a]	3.2±1.1[a]	14.4±3.0[a]
3 months***					
T	23.8±1.9[b]	29.6 ±1.9[a]	1.2±0.6[b]	1.4±0.5[b]	12.8±4.1[b]
MT	23.0±1.3[b]	32.6±1.3[a]	1.0±0.5[b]	1.2±0.6[b]	5.6±2.2[b]
T-HS	28.0±2.1[a]	29.0±3.9[a]	2.6±0.7[b]	2.0±0.4[b]	19.0±5.6[a]
MT-HS	29.4±1.9[a]	32.4±5.2[a]	1.8±0.3[b]	2.4±0.5[b]	19.4±7.2[a]

[a-b] Values with different letters at the same column are significantly differed P<0.05

Each value represents the mean ± SE of six animals.

MT= Methyltestosterone treated fish T = *Oreochromis niloticus* (non-MT treated),

T-HS = Heat stressed *Oreochromis niloticus*, MT-HS = Heat stressed MT treated fish

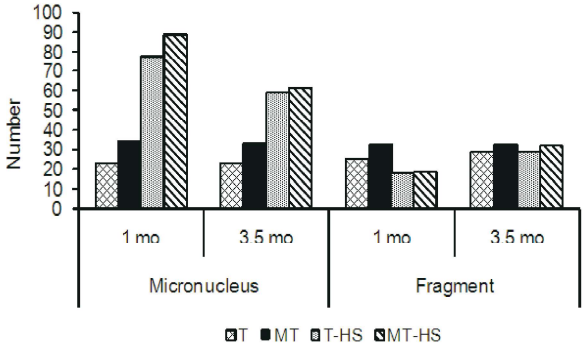

Fig. 2: Comparison in the number of micronucleus and fragments reported in MT and non-MT treated fish at 1 and 3.5 months in the absence and presence of heat stress

Number of chromosomal fragments significantly decreased in heat stressed group 1 age compared with other groups (Table 2). This decrease in the number of fragments associated with significant increase in the number of micronucleated erythrocytes when compared with other groups (Table. 1, Fig. 2)

DISCUSSION

Micronucleus test is one of the simplest short-term tests for biomonitoring the quality of fresh water. In aquatic vertebrates this test can be used to assess the influence of contamination in the aquatic environment [22, 24]. In our study, all fish encountered high number of micronucleated erythrocytes and different types of chromosomal aberrations this may be due to the fish used in this study were obtained from a farm used sewage plant discharge as a source of water. Kusamran *et al.* [25] and Minisii *et al*. [26] demonstrated the induction of micronuclei in fish farm from river polluted by industrial and agricultural effluents.

The induction of micronucleus could be a result of DNA strand breakage leading to a centric chromosome fragment or due to chromosome or chromatid break lagging in anaphase [27]. From our study we could indicate micronuclei as a good tool in measuring genotoxicity in Oreochromis niloticus fish under normal condition and in the presence of heat stress. We suggested that, the heat stressed age one groups, which showed the highest number of micronucleated polychromatic erythrocytes associated with the lowest number of chromosomal fragments may have more extracted chromosomal fragments than the other groups.

There were no significant differences in micronucleus frequencies and DNA damage in Nile tilapia Caused by MT observed by Rivero-Wendt [28].

MT and untreated Nile tilapia showed the same mitotic index at both group ages, under normal and heat stress condition, which indicate that they have the same growth rate. Green and Teichert-Coddington [29], evaluated growth and survival of MT treated and untreated Nile tilapia fry during androgen treatment, nursery and grow-out phases. They found no significant difference in their growth during any phase of production.

No cytogenetic differences were observed between MT and non-MT treated fish at both age groups under normal condition and in the presence of heat stress. The genotoxic effects of MT administration on somatic cell in fish are scarce. However, the effect of administration of MT on sex chromosome synapse (Synaptonemal complex-SC) on *Oreochromis niloticus* was studied [30]. MT did not result in obvious SC lesions,

breakage or a synapsis. We suggested that MT treatment has no genotoxic effect on *Oreochromis niloticus* fish following hormone cessation.

In our study we did not found a significant effect of the heat stress on mitotic activity of the cell. Although, Debec and Marcaillou [31] found that heat shock severely affect mitosis through induction of sever defects in centriol organization. Aleksandrov and Kisliuk [32] demonstrated that heating as well as any other denaturating agent act as an inadequate stimulant, could give rise to synthesis of heat stress proteins. Weigant *et al.* [33], demonstrated that low doses of toxic compounds might under certain condition have beneficial effects related to stimulation of endogenous cytoprotective effective mechanisms through enhancement of synthesis of heat shock protein. Although some of the classes of Hsp's clearly have distinct activities, they also exhibit overlapping functions [34], cooperate in their activities [35-37]. Furthermore, Mcalister and Finkelstein; Plesofsky-Vig and Brambl [38, 39] found that, the ability of the organism to survive severe heat stress is increased if an organism is first exposed to a mild heat treatment. We suggested that exposure of fish to sewage plant discharge act as mild stimulant to HSP proteins which decrease the effect of heat stress on mitotic activity of the cells. Furthermore, Hut *et al.* [17], found that heat stress protein protect the cells from most division abnormalities due to the involvement of heat stress protein in the mechanism of heat-damaged mitotic centrosome.

We have demonstrated that, heat stress is genotoxic, it cause increased number of aberrant cells, many kinds of structural chromosomal aberrations and end-to-end association, which indicate chromosomal stickiness. Anitha *et al.* [19], demonstrated the induction of structural chromosomal aberrations in gold fish by heat shock.

Xiao *et al.* [40], observed an increase in micronucleus rates and DNA damage score associated with increase of HSP70 (the most abundant mammalian HSP) in lymphocytes of coke-oven workers. Furthermore, the individual which had high HSP level showed lower genotoxic damage. However, we observed that the genotoxic effect of heat stress increased in younger age fish (group 1) than the older age fish, they acquire more nuclear anomalies. Murtha and Keller [41] observed a decrease in the expression of heat stress protein (HSP70) in young zebra fish and increased heat shock factor 1a in mature fish, which indicate that heat shock response is detectable in mature fish.

From our study we conclude that, MT treatment has no clastogenic effect on the chromosome of *Oreochromis niloticus*. Heat stress is genotoxic, younger age fish is more affected than older age. Exposures of fish to sewage plant discharge provide adaptive response to the harmful effect of heat stress

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