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FULL LENGTH ARTICLE

Erythrocytes alterations of monosex tilapia (*Oreochromis niloticus*, Linnaeus, 1758) produced using methyltestosterone

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Abstract The present study aims to investigate the effects of methyltestosterone on monosex farmed tilapia, *Oreochromis niloticus* by detection of apoptosis, micronucleus and alterations of erythrocytes. Fishes were obtained from four localities (Assiut as a control and Beheira, Alexandria and Kafr EL-Sheikh; three farms from each governorate as farmed monosex produced using methyltestosterone). Blood smears were processed for Hematoxylin and eosin technique. The major alterations recorded in the red blood cells were as swelled cells (Sc), tear drop-like cells (Tr), and sickle cells (Sk). Also, a significant difference ($P \leqslant 0.001$) between three governorates and Assiut was recorded in the micronucleus test, apoptosis and altered erythrocytes. These alterations are considered as an indication for performance and health of fish in the monosex culture medium indicating the side effects of overdose induction of MT.

Introduction

Tilapia fish was considered as one of the most important fish of all aquaculture in 21st century (Fitzsimmons, 2000; Sawhney and Johal, 2000). Tilapia has certain favorable characteristics, like most tolerant to adverse environmental conditions, and can survive at low dissolved oxygen (Magid and Babiker, 1975; Ross, 2000), euryhaline (El-Sayed, 2006), relatively fast growth and efficient food conversion (Asad et al., 2010).

The production of tilapia (*Oreochromis* sp.) all-male populations is important in aquaculture to avoid energy consumption in reproduction and to produce the sex with the larger growth potential (Macintosh and Little, 1995; Green et al., 1997; Dan and Little, 2000; Tran-Duy et al., 2008). For producing mono-sex populations the steroid-induced sex inversion such as 17α-methyltestosterone (MT) are the most common techniques. The use of those techniques is widespread in tilapia aquaculture (Green et al., 1997; Green and Teichert-Coddington, 2000; Wahby and Shalaby, 2010; Celik and Guner, 2011), but the side effects of the overdoses of those hormones are not reported especially in the field studies.

The micronucleus (MN) test has been used successfully as a mutagenic assay in fish (Minissi et al., 1996; Kan et al., 2012). Therefore, the MN test in fish erythrocytes has been used as a

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biomarker for environmental mutagenesis (Al-Sabti and Metcalfe, 1995; Ateeq et al., 2002; Kan et al., 2012). The detection of micronucleus (MN) helps us to know the status of water quality, the health of species and potential risk (Al-Sabti and Metcalfe, 1995; Mekkawy et al., 2011). The study of micronucleus test and erythrocytes alterations in fishes by various chemicals has been reported (Grisolia and Starling, 2001; Ferraro et al., 2004; Talapatra and Banerjee, 2007; Mekkawy et al., 2011). Also, its feasibility has already been established in *Clarias gariepinus* (Ateeq et al., 2002; Mekkawy et al., 2011; Sayed et al., 2013). It has been reported that, nuclear abnormalities are a bioindicator of genotoxic damage in fish (Bombail et al., 2001; Talapatra and Banerjee, 2007; Mekkawy et al., 2011; Sayed et al., 2013). Alternatively, various erythrocytes alterations are effective indicators of cytotoxicity (Mekkawy et al., 2011; Sayed et al., 2013; Sayed and Fawzy, 2014; Ateeq et al., 2002). Cell shrinkage, membrane blebbing and chromatin condensation are signs for apoptotic cells (Talapatra and Banerjee, 2007; Iarmarcovai et al., 2008) and that have been considered as indicator of abnormal cell divisions (Cavas and Ergene-Gozukara, 2005a; Talapatra and Banerjee, 2007).

Therefore, the aim of the present work was undertaken to investigate the side effects of methyltestosterone in sex reversal in monosex farms of Nile tilapia in Egypt using biomarkers tools as apoptosis detection, micronucleus and morphological alterations in blood erythrocytes.

Material and methods

Sample collection

Seventy-two male fishes of The Nile tilapia, *Oreochromis niloticus* were caught from Assiut farms as control and monosex fishes from three farms of Beheira, Alexandria and Kafr EL-Sheikh as monosex farms in Egypt. The site and data of those farms and fishes were reported in our previous publication (Sayed and Moneeb, 2015).

Water quality assessment

Water-quality criteria [pH, dissolved oxygen, water temperature, conductivity, salinity, turbidity, phenols, chloridate, fluoridate, sulfate, nitrate, cyanide and ammonia] of the selected sites were measured during the fish collection. Total Fe, Cd, Pb, Zn, Cr, and Hg were measured using graphite furnace AA (GFAA) spectroscopy. Water sampling and quality assessment were done according to APHA, 2005. In addition methyltestosterone concentrations were estimated in water and sediments of the farms in concern according to the protocol of Risto et al. (2013) using kits purchased from R-Biopharm AG, Darmstadt, Germany.

Determination of methyltestosterone in fish serum and muscle

Estimation of the MT concentrations in fish serum and muscle, was prepared according to the protocol of Risto et al. (2013) using kits purchased from R-Biopharm AG, Darmstadt, Germany.

Micronucleus test and erythrocytes alterations

Six fishes from each farm were removed and anesthetized in MS-222 (50 mg/l) solution for blood smearing. Peripheral blood samples were obtained from caudal vein; three air dried blood smears for each fish were prepared. Fixation, dehydration, staining and clearing were done according to Pascoe and Gatehouse, 1986. Examination and criteria for identifying and scoring MN were done according to Al-Sabti and Metcalfe, 1995; Schmidt, 1975.

Apoptosis detection

Apoptotic erythrocytes were detected using Acridine Orange (AO) stain (Cat. No. A1031), Life Technologies, 5791 Van Allen Way Carlsbad, CA 92008 USA). The modified protocol according to Darzynkiewicz, 1990 was used to detect the apoptosis in RBCs, after preparation of blood smears on clean glass slides, the slides were washed in 1× PBS (pH = 7.2). AO buffer (17 μg/l Acridine Orange in 1× PBS buffer) was added to the slides for 30 min in the dark. Decolorization process was achieved by washing the slides every 30 min with 1× PBS for four times. Fixation was in paraformaldehyde 4% for 5 min. Finally observation of cells was made under Zeiss Axioplan2 fluorescence microscope (×200) provided with a digital 3 CCD color video camera (Sony, AVT-Horn).

Statistical analysis

Data statistical analysis was done using the statistical package for the social science; Inc., Chicago, IL, USA (SPSS, 1998) statistical program, version 16.

Ethical statement

The study was carried out in accordance with the Egyptian laws and University guidelines for the care of experimental animals. All procedures of the current work have been approved by the Committee of the Faculty of Science of Assiut University, Egypt.

Results

Physicochemical water parameters

The measured physico-chemical parameters of the water samples collected from Assiut farms as control and three farms of Beheira, Alexandria and Kafr EL-Sheikh as monosex farms were reported in Sayed and Moneeb (2015). Most of these parameters showed the highest values in the water of the monosex farms in comparison to Assiut farms. In addition, Table 1 shows mean ± SE of methyltestosterone concentrations in water and sediments collected from Assiut farms as control and the three monosex farms. The three monosex farms showed highest values in comparison to Assiut farms which showed no detection (< 0.1).

Determination of methyltestosterone in fish serum and muscles

Table 1 shows mean ± SE of methyltestosterone concentrations in serum and muscle fish samples from Assiut farms as

Table 1 Methyltestosterone concentrations in water, sediment, serum and muscle from Assiut and monosex farms, (n = 6).

Governorates	Water (ng/ml)	Sediment (ng/g)	Serum (ng/ml)	Muscle (ng/g)
Assiut	<0.1	<0.1	<0.1	<0.1
Alexandria	0.87 ± 0.11	0.33 ± 0.05	2.32 ± 0.31	1.54 ± 0.15
Kafr EL-Sheikh	1.07 ± 0.07	0.35 ± 0.04	2.03 ± 0.16	0.93 ± 0.07
Beheira	1.28 ± 0.1	0.67 ± 0.04	3.47 ± 0.19	2.01 ± 0.19

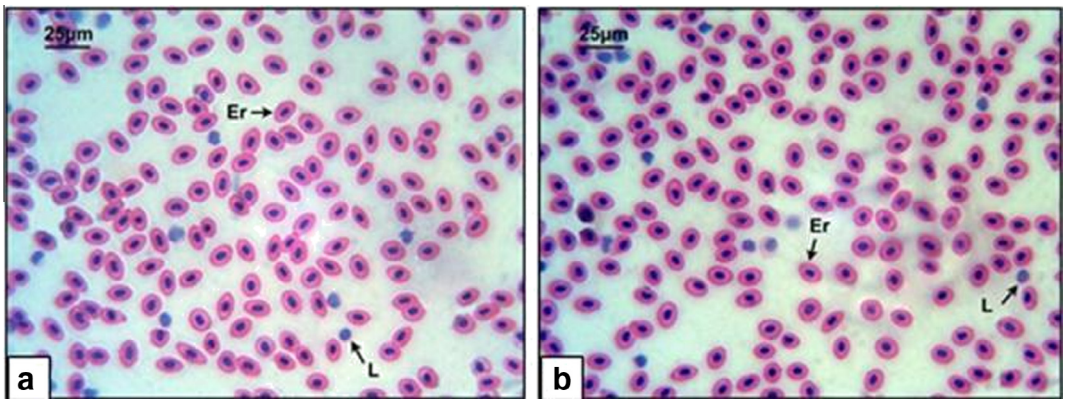

Figure 1 (a, b) Blood film of Nile tilapia *Oreochromis niloticus* from Assiut farms showing normal erythrocytes (Er) and leucocytes (L). (H&E, ×400).

control and three farms of Beheira, Alexandria and Kafr EL-Sheikh as monosex farms. The three monosex farms showed highest values of MT concentrations in comparison to Assiut farms in which no values in water, sediments, serum and muscle were recorded (<0.1).

Apoptosis detection and erythrocytes alterations

Fig. 1 shows the normal structure of erythrocytes (Er) and leucocytes (L) of the blood smear of tilapia in Assiut farms. Figs. 2–4 show the blood smears of tilapia from Beheira, Alexandria and Kafr EL-Sheikh farms, respectively. They represent the normal structure of erythrocytes (Er) and leucocytes (L) with presence of some alterations of RBCs in the studied fishes. The major alterations of RBCs were swelled cells (Sc), tear drop like cells (Tr), their shape looks like tear with pointed apices and sickle cells (Sk) which vary in shape between ellipsoidal, boat-shaped and genuine sickles. Also, hemolyzed cells (Hc) and cells have prominent vacuoles (Va) which were observed in the blood smears.

The apoptotic cell percentage appears in the monosex farms more than the control fish from Assiut farms. Also Fig. 5 shows the apoptotic cells of Assiut farms lower than the monosex farms which appeared in light green color under the fluorescence microscope stained with Acridine Orange.

Variation in apoptotic RBCs, micronucleus and altered erythrocytes count

The apoptotic cell percentage of control fish from Assiut farms was 0.87 ± 0.12% and this percentage increased significantly (P < 0.05) in the monosex farms (Alexandria, Kafr EL-Sheikh and Beheira). Also, the micronuclei percentage of control fish from Assiut farms was 0.09 ± 0.04% and this value was increased significantly (P < 0.05) in the monosex farms. The number of altered erythrocytes of control fish from Assiut farms was 2.56 ± 0.2% and this value was also significantly increased (P < 0.05) in the monosex farms (see Fig. 6).

Discussion

The synthetic hormone, 17α-methyltestosterone (MT), is used to induce male monosex in fish hatcheries with different types of manipulation and techniques (Wahby and Shalaby, 2010; Celik and Guner, 2011). By feeding small amounts of male hormone to tilapia fry before and during sexual differentiation, virtually all the treated fish develop as males morphologically and the potential of the stock to reproduce is thereby eliminated.

This form of sex control has the added benefit that male tilapias generally grow faster than females, with a result that all-male fish are larger and more uniform in size than mixed sex tilapias (Smith and Phelps, 1997; Hussain et al., 2005). These desirable growth characteristics are particularly shown by MT treated Nile tilapia) *O. niloticus*), which is the major tilapia species cultivated commercially worldwide (FAO, 2006).

To our knowledge this is the first field investigation dealing with the study of the effects of use of methyltestosterone in tilapia sex reversal at Egypt in the field not experimentally. The detailed investigation of water-quality assessment along the studying areas showed similar mean values of nearly all

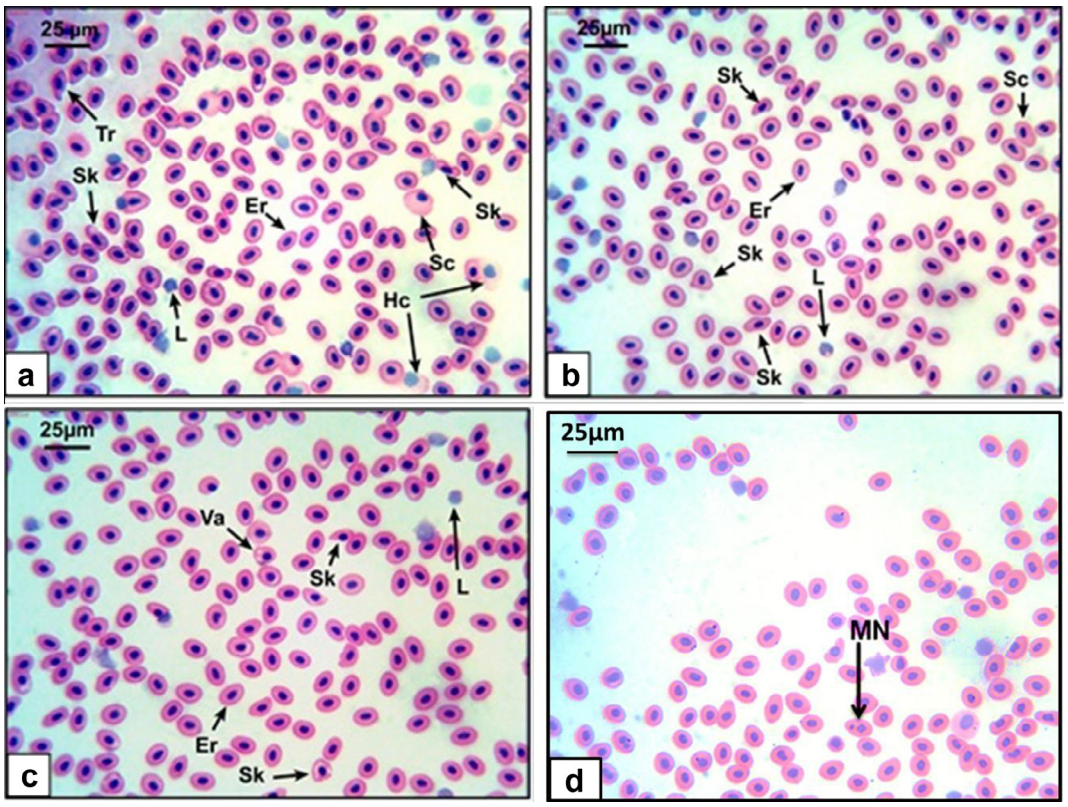

Figure 2 (a–d) Blood film of Nile tilapia *Oreochromis niloticus* from three farms at Beheira showing normal erythrocytes (Er) and leucocytes (L) with presence of swelled cells (Sc), sickle cells (Sk), tear drop like cells (Tr), hemolyzed cells (Hc), micronucleus (MN) and cells have prominent vacuoles (Va) (H&E, ×400).

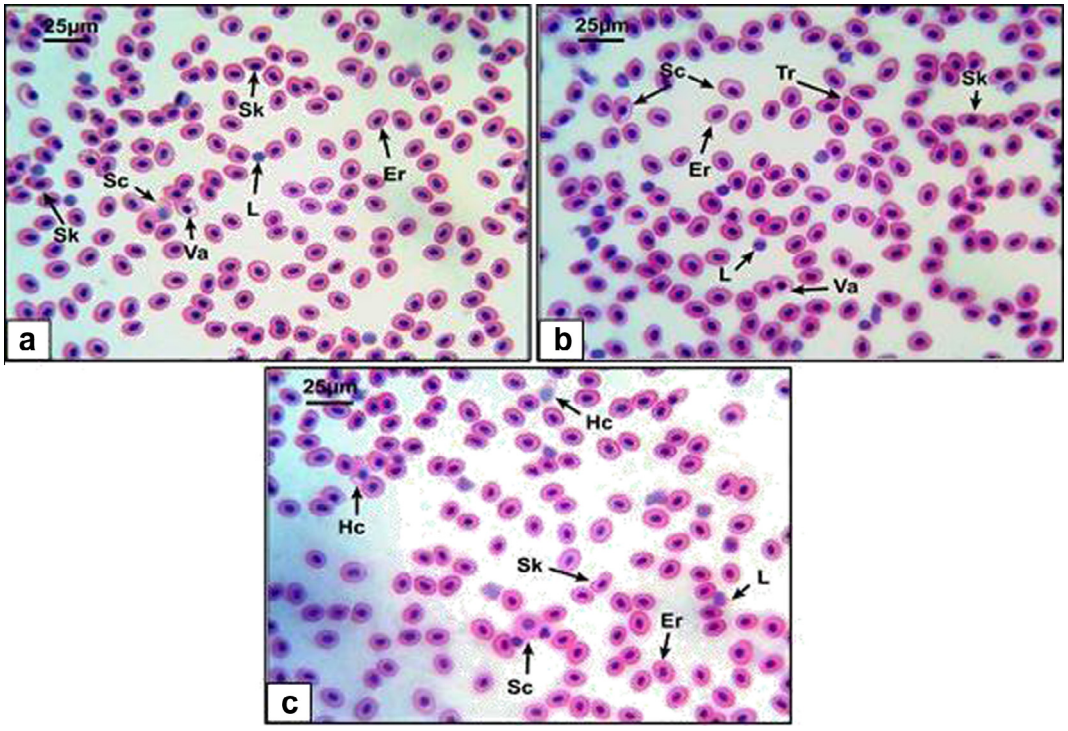

Figure 3 (a–c) Blood film of Nile tilapia *Oreochromis niloticus* from three farms at Alexandria showing normal erythrocytes (Er) and leucocytes (L) with presence of swelled cells (Sc), sickle cell (Sk), tear drop like cells (Tr), hemolyzed cells (Hc) and cells have prominent vacuoles (Va) (H&E, ×400).

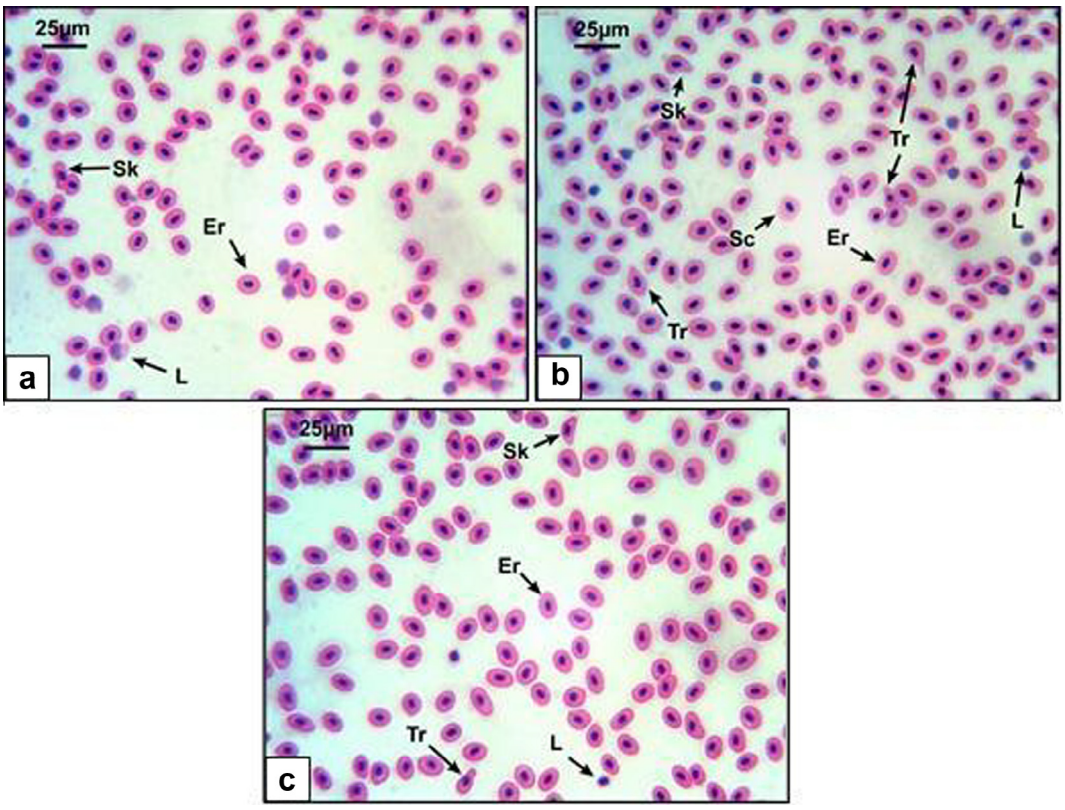

Figure 4 (a–c) Blood film of Nile tilapia *Oreochromis niloticus* from three farms at Kafr EL-Sheikh showing normal erythrocytes (Er) and leucocytes (L) with presence of swelled cells (Sc), sickle cells (Sk), tear drop like cells (Tr), hemolyzed cells (Hc) and cells have prominent vacuoles (Va). (H&E, ×400).

the detected physico-chemical parameters in water collected from farms. Those results indicated that the physico-chemical parameters of water which was collected from the fish farms of the selected areas is high compared with those collected from river Nile in a previous study (Osman et al., 2012). The results of Osman et al. (2012) proved the presence of organic and inorganic pollutants in water along the river Nile and this finding indicated the fact that the level of contamination in the river Nile is greater than in fish farms.

MN test is used to detect the biological impacts of water pollution (Minissi et al., 1996) and to assess the genotoxicity of chemical compounds after direct or indirect exposure in fishes (Mekkawy et al., 2011; Osman et al., 2012). For fish species, the MN test is an excellent biomarker for cytogenetic studies in (Hooftman and Vink, 1981) and for detecting clastogenic substances in aqueous media in different types of fishes (Abdul-Farah et al., 2003; Cavas and Ergene-Gözükara, 2005b). Abdul-Farah et al. (2003) reported time dependent increase in MN induction in the peripheral blood of *Channa punctatus* exposed to pollutants. Also, Kumar et al. (2010), investigated the genotoxic effect of malathion in peripheral erythrocytes of *C. punctatus* and reported that MN frequency increased compared to the control. Our previous studies indicated that the MN frequency in *C. gariepinus* was reported to increase due to the exposure of 4-nonylphenol with concentrations (Mekkawy et al., 2011). It was reported that the increase of MN frequency after gamma irradiation in medaka fish with concentrations and durations. Könen and Cavaş (2008) reported an increase in MN frequency after exposure of trifluralin, and Treflan in the *O. niloticus*. As response to atrazine, Cavas (2011) reported an increase in the induction of MN in peripheral blood of *Carassius auratus*. In the present study the micronucleus percentage is high in the monosex tilapia farms in comparison with the control. These results may be due to the genotoxicity caused by the MT use in sex reversal process especially the results which indicated the high quality of water.

In the present study, methyltestosterone showed some alterations (teardrop-like cells, sickle cells, swollen cells and vacuolated cells) in erythrocytes of fishes from monosex farms in comparison with the control farms. Such alterations have been reported in previous studies in response to 4-nonylphenol (Mekkawy et al., 2011), hypoxic conditions (Sawhney and Johal, 2000), toxicants (Buckley et al., 1976), pesticides (Adeyemo, 2007; Adedeji et al., 2009), gamma radiation (Sayed et al., 2014), UVA exposure (Sayed et al., 2007, 2013), and heavy metals (Oloade and Oginni, 2010; Adeyemo, 2007). Unequal distribution of hemoglobin is the cause of vacuoles observed in erythrocytes (Ateeq et al., 2002; Mekkawy et al., 2011; Bushra et al., 2002). The swelled blood cells were recorded as signs of necrosis (Bushra et al., 2002).

Rodriguez-Cea et al. (2003) reported that fish species are more sensitive to genotoxic pollutants than others. In the present work, Nile tilapia showed a higher degree of DNA damage in the fish sampled from monosex farms observed in the significant increase in micronuclei and the percentage of apoptotic cells. These results are in line with the previous work

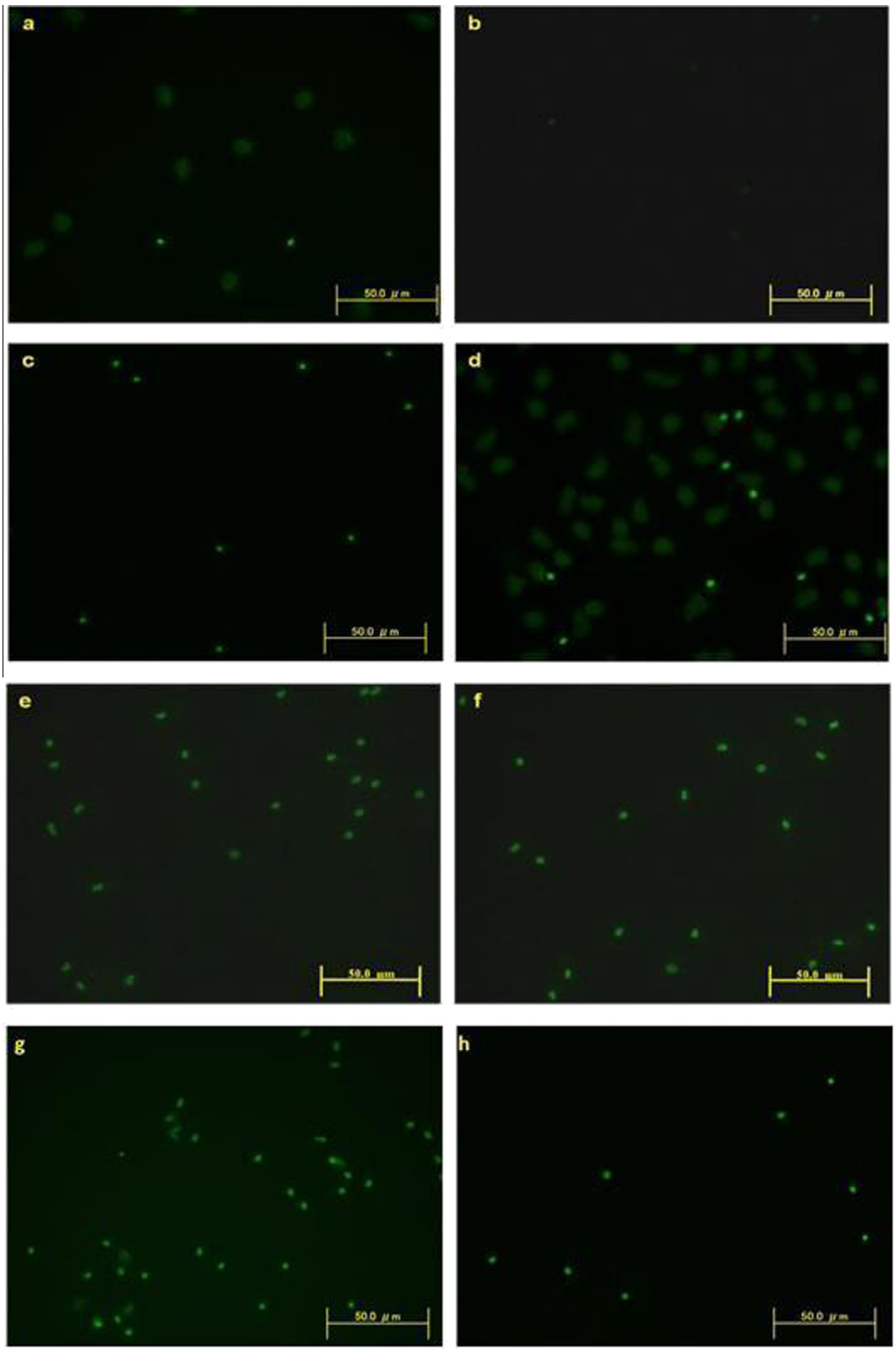

Figure 5 (a,b) The apoptotic cells of Assiut farms, (c,d) Alexandria farms, (e,f) Beheira farms and (g,h) Kafr EL-Sheikh farms in light green color under the fluorescence microscope stained with Acridine Orange.

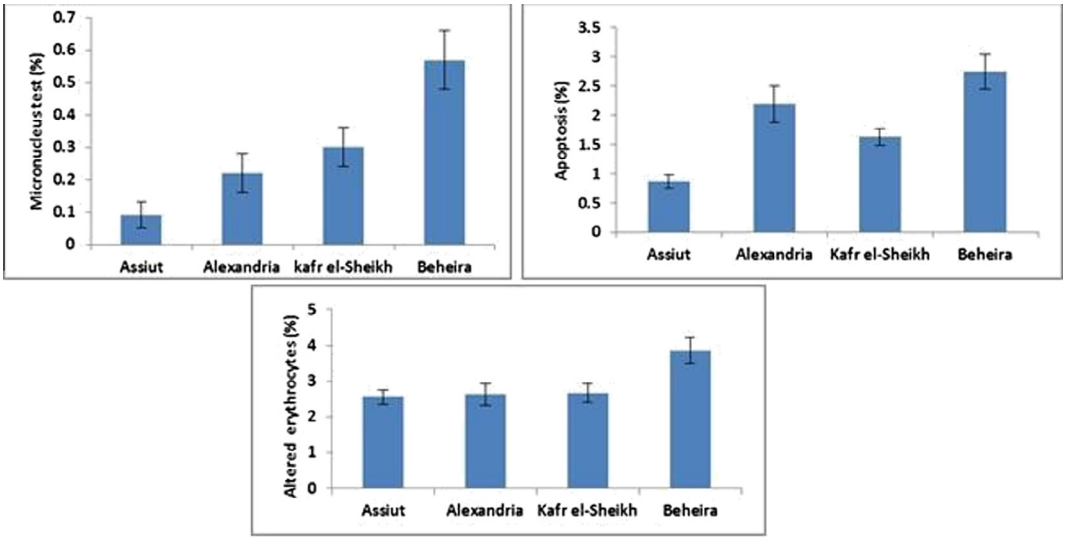

Figure 6 Apoptotic, micronuclei and altered erythrocytes of Nile tilapia from Assiut and monosex farms. Data are presented as the mean ± SE, (n = 6).

(Osman et al., 2012), where a significant increase was found in the frequencies of micronuclei in the blood of Nile tilapia collected from the contaminated sites. The increased apoptosis induction was in accordance with that obtained by (Christen et al., 2013) after exposure to Ag-nanoparticles. Also, Khalil et al. (2011) studied the molecular changes of the synthetic steroid 17α-methyltestosterone on the liver of Nile tilapia; *O. niloticus* and found that MT was able to induce DNA fragmentation and molecular genetic variability.

In conclusion, the estimation of genotoxic effects is may be due to MT use in fish farming. Although based on a relative small data set, our study confirmed high sensitivity of Nile tilapia on using methyltestosterone as indicated by high apoptosis and micronuclei.

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