



Mutagenic effect of the sex reversal hormone Diethylstilbestrol in the Nile tilapia (*Oreochromis niloticus*, L) through sister chromatid exchange analysis

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ABSTRACT

The current study was conducted to explore the mutagenic effect of DES hormone on the treated Nile tilapia chromosomes using sister chromatid exchange (SCE) analysis. Two concentrations of DES hormone (100mg DES/kg and 200mg DES/kg diet) were addressed. The fry were fed for 60 days at 15% of the body weight daily. Two cell replication cycles, in the presence of 5-bromodeoxyuridine (BrdU) were achieved, and intestine tissues were used for chromosomes preparation. For slides staining, acridine orange (AO) was added, and the fluorescent microscope was used for the observation of AO fluorescence of stained slides. The obtained results indicated that the frequency of SCEs per cell and the percentage of cells exhibiting three SCEs per cell increased with the increasing hormone concentration. The present findings confirmed the mutagenic effect of DES hormone and clarified the possibility of using *O. niloticus* as an *in vivo* system for detecting mutagenic and carcinogenic chemicals. In addition, it is recommended not to use the hormone directly in the food served for humans. However, it can be used in the induction of fish sex inversion to XY females and followed by progeny testing, to generate super male YY.

INTRODUCTION

The Nile tilapia *Oreochromis niloticus* is the sixth most cultivated species in the world and it is responsible for more than 99.2% of the world's tilapia production (Reantaso, 2017; Lazaro-Velasco *et al.*, 2019; Hashem *et al.*, 2020; Heiba *et al.*, 2021).

A major problem in tilapia breeding programs is that the female grows slower than the male, in addition to the early sexual maturation and the uncontrolled reproduction. Monosex progeny production upon super male YY is known as the most effective solution to resolve the problems related to mixed-sex or females' breeding (Mair *et al.*, 1997; Gennottea *et al.*, 2012; Hashem *et al.*, 2019).

There are several approaches used for YY production programmes. One approach is through diet containing an estrogen hormone, diethylstilbestrol (DES) induced for fish

sex inversion, followed by selective breeding. **Mair *et al.* (1997)** was the first to use DES hormone to induce sex reversal of male to female followed by progeny testing to generate novel 'YY' male genotypes. Several studies proposed that the DES hormone has mutagenic and carcinogenic properties, which raised widespread public health concerns (**Yang *et al.*, 2013**).

The international organizations concerned with humans health prohibit the use of DES hormone or other synthetic hormones in the food that will be consumed by human. However, it was recommended that using hormones for sexual inversion should be established by the governmental framework of the country that will use this technique for human and environmental safety (**Celia *et al.*, 2018**).

In addition, *O. niloticus* is the most public freshwater fish species used in the genotoxic effects studies of the environmental factors (**Hamdoon *et al.*, 2002**, **Hashem *et al.*, 2019**; **Heiba *et al.*, 2021**).

On the other hand, among the cytogenetic and molecular biological markers, a sister chromatid exchange (SCE) analysis is considered to be one of the most sensitive methods usually used to evaluate the genotoxic effects of different environmental factors (**Maisels & McDonagh, 2008**; **Kwasniewska & Bara, 2020**). SCE is an exchange in sister chromatids during DNA replication. It may happen as a normal feature of cell division. It does not cause any change in the chromosome structure, but its incidence increases when a cell's DNA is damaged by a mutagenic factor such as radiation and chemicals (**Wilson & Thompson, 2007**; **Kanmaz *et al.*, 2017**; **Heijink *et al.*, 2021**). SCE has been considered a biomarker for mutagenicity of sex reversed hormone used for sex reversal induction in the Nile tilapia (**Hamdoon *et al.*, 2002**). It is considered a sensitive indicator of the genome's instability (**Wietmarschen & Lansdorp, 2016**). Furthermore, SCE is a very sensitive marker for genotoxic stress detection during DNA replication (**Sunada *et al.*, 2019**).

Thus, this study focused on exploring the mutagenic effect of DES hormone on the treated Nile tilapia chromosomes by using sister chromatid exchange analysis.

MATERIALS AND METHODS

Diet preparation:

Two treatment diets (30% protein) with Diethylstilbesterol hormone (DES, Sigma Aldrich Chemical Co., USA) were prepared as discribed in the studies of **Guerro (1975)** and **Hamdoon *et al.* (2013)** through the alcohol evaporation method.

Fish samples:

The fry with a total length range of 9-12mm were collected from the adult females from the Fish Breeding Lab., Animal Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Egypt. The fry were divided into 3 groups of 100 individuals each. One group was used as a control and fed on a diet without hormone. The other two groups were used for hormone treatments. One

was used for the 100mg DES/kg diet treatment and the last group for the 200mg DES/kg diet. The fry were fed for 60 days at 15% of the body weight daily before being sacrificed by decapitation and chromosomes' preparation.

Tissues preparation:

To obtain metaphases exhibiting sister chromatid differentiation, two cell replication cycles, in the presence of 5-bromodeoxyuridine (BrdU), were achieved as described in **Hamdoon et al. (1996, 2002)**, with some modifications. Fish were injected every 48 hours with 0.5mg/g of body weight of BrdU (Sigma chemicals) three times. It was dissolved in phosphate buffer saline (PBS) and sterilized by filtration before injection. After six days, fish were injected intraperitoneally (I.P.) with 0.1% colchicine dissolved in PBS (10µl/g of body weight), mixed with 10µl/g of ethidium bromide solution (500µg/ml). After four hours, intestine tissues were removed after decapitation, minced into 0.5cm pieces and placed in Petri dishes containing 0.4% KCl (hypotonic solution) for 30min. Then, tissues were fixed in cold freshly fixative (3 methanol: 1 acetic acid) and changed after one hour. The tissues were kept in the fixative in small bottles (10 ml) until slides preparations.

Slides preparation:

Slides were prepared by using the solid tissue technique as described in **Kligerman and Bloom (1977)**. For slides staining, acridine orange (AO) was used as described in the studies of **Schneider et al. (1978)** and **Hamdoon et al. (1996)**. The fluorescent microscope was used for the observation of AO fluorescence of stained slides. For permanent preparation, the slides were destained and restained with Giemsa stain following the procedure of **Hamdoon et al. (1996)**.

At the end of the experiment, the means of SCE rate per cell and percentages of cells displaying SCEs were counted.

RESULTS

Excellent sister chromatid differentiation was obtained using the incorporation and detection of 5-bromodeoxyuridine (BrdU) enabled in Nile tilapia chromosomes. The chromatids can be differentiated within one chromosome, and hence, the SCEs were seen with great clarity and resolution. The chromosomes of metaphase of an intestinal tissue cell presenting sister chromatid differentiation with SCE are shown in Fig. (1).

Metaphase chromosomes stained with Giemsa and exhibit sister chromatid differentiation with SCE are observed in Fig. (2a) and normal metaphase without sister chromatid differentiation or SCE is displayed in Fig. (2b).

The mean of SCE incidences per cell counted in the intestine tissue of the untreated fish as well as fish treated for 60 days with DES hormone levels of 100 mg and 200 mg/kg diet are presented in Table (1).

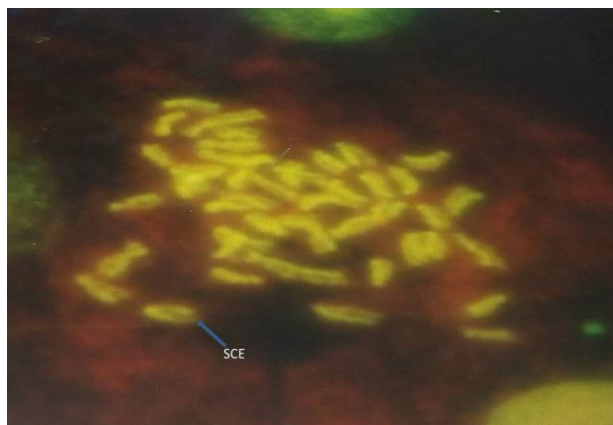
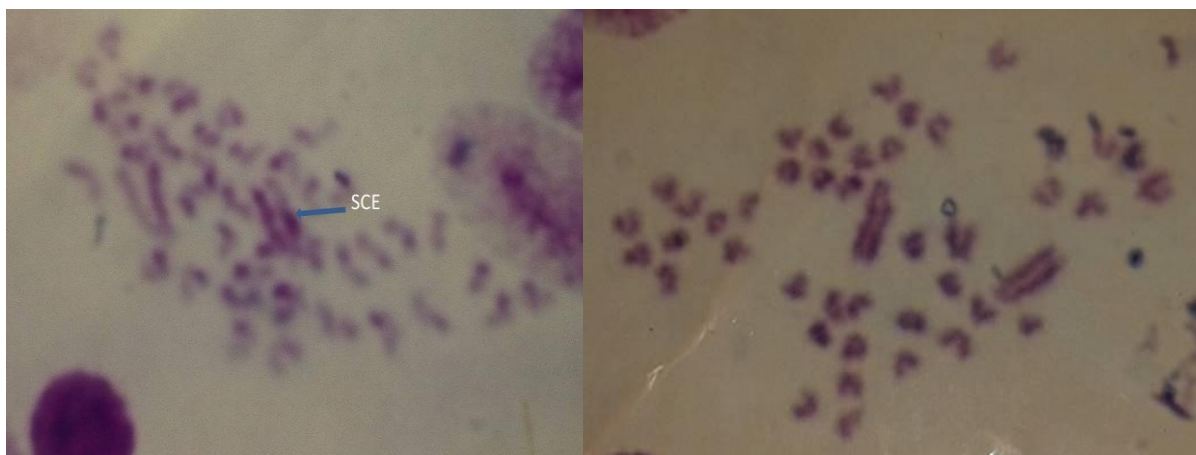


Fig. 1. Metaphase chromosomes with sister chromatid exchange (SCE) in the intestinal tissue cell of the Nile tilapia stained with acridine orange (AO)



(a)

(b)

Fig. 2. Metaphase chromosomes in the intestinal tissue cell of the Nile tilapia stained with Giemsa showing:

(a) Metaphase chromosomes with sister chromatid exchange (SCE).

(b) Normal metaphase chromosomes without sister chromatid differentiation.

Table 1. Means of sister chromatid exchanges (SCEs) per cell and cells with SCEs percentage in fish cells treated with different concentrations of DES hormone

Treatment	No. of examined cells	No. of cells with SCEs	No. of scored SCEs	Means of SCEs per cell	Percentage of cells with SCEs
0 mg DES/kg diet (Control)	252	84	87	0.345	33.33
100 mg DES/kg diet	270	150	186	0.689	55.56
200 mg DES/kg diet	250	160	240	0.960	64.00

Obtained results display a spontaneous SCE per cell frequency of 0.345 in untreated fish with DES (control). In the treated fish, the means of SCE per cell were increased, reaching 0.689 and 0.960 in the fish treated with a 100 and 200 mg DES/kg diet, respectively.

In untreated fish, the percentage of cells with SCEs was 33.33%. The percentage of cells exhibiting SCEs increased to 55.56 and 64.00% when the fry were fed on DES-treated diet at 100 and 200mg/kg levels, respectively. The highest level of hormone (200mg/kg diet) almost doubled the percentage of cells displaying SCEs.

Table (2) shows SCEs distribution in control fish and those treated with both levels of DES hormone.

Table 2. No. of cells with sister chromatid exchanges (SCEs) and percentage of SCEs No. distribution in fish cells treated with different concentrations of DES hormone

Treatment	No. of cells with SCEs	Percentage of SCEs No. distribution		
		1	2	3
0 mg DES/kg diet (Control)	84	62 (73.81%)	22 (26.19%)	0 (0.00%)
100 mg DES/kg diet	150	99 (66.00%)	48 (32.00%)	3 (2.00%)
200 mg DES/kg diet	160	106 (66.25%)	36 (22.50%)	18 (11.25%)

Results indicate that, the percentages of cells displaying SCEs increased with increasing DES levels compared to the control. The percentage of cells exhibiting three SCEs per cell was threefold in the 100mg DES treatment compared with the control. Whereas, the 200 mg DES level caused an eighteen-fold and six-fold increase in this percentage, compared to the control and 100 mg DES treatment, respectively. These results show the frequency of higher percentages of cells with high SCE frequencies based on DES hormone treatment.

DISCUSSION

Different studies have used different concentrations of DES hormone for the feminization of the Nile tilapia. **Hamdoon *et al.* (2013)** used 100 and 150mg/kg diet; whereas, **Marín-Ramírez *et al.* (2016)** used 100, 200, 300 and 400 mg kg/kg diet. In the present study, 100 and 200mg DES/kg diet were used to study the effects of hormone on the Nile tilapia chromosomes through sister chromatid exchanges (SCEs) analysis. Sister

chromatid exchanges were used as a sensitive assay for chromosomal damage recognition, and the cumulative SCE incidence happens with exposure to genotoxic agents (Kanmaz *et al.*, 2017; Kwasniewska & Bara, 2020). In the present study, 5-bromodeoxyuridine (BrdU) was used to obtain sister chromatid differentiation instead of other analogs. Based on most new studies by using Strand-seq, which is a single-cell DNA strand sequencing technique, no effect of the BrdU was present with variable concentrations, on SCE occurrence in either normal or bloom syndrome cells (Wietmarschen & Lansdorp, 2016). Based on this principle, SCE reflects the DNA repair actions that occur spontaneously (Kwasniewska & Bara, 2020). Therefore, in this investigation, SCE analysis was preferred as a method to estimate the mutagenic effect of DES hormone compared to previous studies. The frequency of sister chromatid exchange (SCE) assessed in the intestine of the control fish was 0.345, which is very close to those described in *O. niloticus* in the studies of Hamdoon *et al.* (1996) and Hamdoon *et al.* (2002). The higher SCE frequency of DES-treated fish, as compared to untreated ones, showed that DES is an effective SCE inducer and has genotoxic effects on the Nile tilapia fish that can be used as a bioassay. Rudiger *et al.* (1984) postulated that, DES (identified as carcinogenic) can cause SCE in human cultured fibroblasts. The model of the mouse, which imitates many DES-related effects that were seen in humans, proposes that prenatal DES exposure causes changes that may affect the subsequent offspring generation (Titus-Ernstoff *et al.*, 2009). Several studies proposed that the DES hormone has mutagenic and carcinogenic properties, which raised widespread public health concerns (Yang *et al.*, 2013).

The occurrence of SCE depends on the cell's potential to correctly repair the damage of DNA and eliminate lesions prior to undergoing the replication of DNA (Mourelatos, 1996; Wilson & Thompson, 2007). It is essential for cells to pass through the S-phase before the DNA damage results in the development of SCE. If there is no repair of induced lesions within the two cell cycles before sampling, these lesions will contribute to the development of SCE in both the first and second cycles. In the present search, DES treatment might affect the effectiveness of the DNA damage repair in *O. niloticus* intestinal cells.

The greatest rates of SCEs obtained in the intestinal tissues of *O. niloticus* as a response to DES treatments were mainly attributed to the elevated rate of cells with more than one SCE. In the present work, the results show a predominance of cells displaying three SCEs in both DES concentrations compared to the control. The low DES concentration (100mg/kg diet) is less harmful than the higher one (200mg/kg diet). The results of Lanza *et al.* (1999) showed a higher occurrence of the cells with high SCE incidences in cancer patients, following the management of high doses of cytotoxic drugs, which supports the finding results in this investigation.

For aquatic environments, only a few SCE test systems are available (Harrison & Jones, 1982; Hamdon *et al.*, 2002). However, the use of hormones for sexual inversion

should be established by the governmental framework, and extreme care must be taken when using these hormones for the least possible environmental influence and to assure food and human health safety, rendering sustainability in the fish sector (Celia *et al.*, 2018).

In conclusion, the obtained results proved the mutagenic effect of DES hormone and clarified the possibility of using *O. niloticus* as an *in vivo* system for detecting the mutagenic and carcinogenic chemicals. It is worth noting that, the hormone must not be used directly in the food that will be used by humans. On the other hand, it can be used in the induction of fish sex inversion to XY females and followed by progeny testing to generate super male YY.

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