

Genotoxic Effects in Erythrocytes of *Oreochromis niloticus* Exposed to Nanograms-per-Liter Concentration of 17 β -Estradiol (E₂): An Assessment Using Micronucleus Test and Comet Assay

Graziela Sponchiado · Eliana Mara Fortunato de Lucena Reynaldo · Any Caroline B. de Andrade · Eliane Carvalho de Vasconcelos · Mônica Lucia Adam · Cíntia Mara Ribas de Oliveira

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Abstract Pharmacologically active substances used in the treatment of human and animal illnesses may usually enter the aquatic environment via effluents from sewage treatment plants, as they are not completely biodegraded or removed during waste water treatment. 17 β -Estradiol genotoxicity was evaluated in *Oreochromis niloticus* (family Cichlidae) using micronucleus test, other nuclear abnormalities assessment, and the comet assay with erythrocytes. Fish were exposed to aqueous systems contaminated with 6 ng/L 17 β -estradiol for three periods: 24 h, 48 h, and 10 days. The results showed that 17 β -estradiol has genotoxic potential in different periods, since significant differences ($P=0.036$) were observed in the micronucleus frequencies of the 10-day exposure groups in relation to the control group. Also, the same was

observed when comparing the nuclear abnormality frequencies ($P=0.018$) of the 24-h exposure group with the negative control group, and when using comet assay ($P<0.001$) for 48-h evaluations. The tested concentration of the 17 β -estradiol gave rise to mutagenic and genotoxic effects on the blood cells of *O. niloticus*, therefore the substance being considered a clastogenic chemical contaminant on both acute and chronic exposures. The assessment using a combination of micronucleus test, nuclear abnormalities, and comet assays proved to be suitable and useful in the genotoxicity testing of 17 β -estradiol at nanograms per liter.

Keywords Genotoxicity · 17 β -Estradiol · Micronuclei test · Alkaline comet assay · Pharmaceutical · Fish

G. Sponchiado · E. M. F. de Lucena Reynaldo · E. C. de Vasconcelos · C. M. R. de Oliveira (✉)
Mestrado Profissional em Gestão Ambiental,
Universidade Positivo,
Prof. Pedro Viriato Parigot de Souza Street, 5300,
81280-330 Curitiba, Paraná, Brazil
e-mail: cmara@up.com.br

G. Sponchiado
e-mail: graziela_s@hotmail.com

G. Sponchiado
Grupo de Pesquisa em Farmacologia e Fisiopatologia da pele, Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Paraná, UFPR, Curitiba, Paraná, Brazil

A. C. B. de Andrade · E. C. de Vasconcelos · C. M. R. de Oliveira
Núcleo de Ciências Biológicas e da Saúde,
Curso de Farmácia,
Universidade Positivo,
Curitiba, Paraná, Brazil

M. L. Adam
Núcleo de Biologia, Centro Acadêmico de Vitória de Santo Antão:UFPE,
Vitória de Santo Antão, Pernambuco, Brazil

M. L. Adam
Programa de Pós-Graduação em Ciências Biológicas,
Centro de Ciências Biológicas, UFPE,
Recife, Pernambuco, Brazil

1 Introduction

Significant quantities of human and veterinary pharmaceuticals are excreted by urine and excrement mainly on their original structures but also as their metabolites. Most of such medicines are not completely biodegraded, and due to their partial removal during waste water treatment (WWT) and water treatment, these contaminants may be detected in aquatic systems. In many countries, low levels of pharmaceuticals have been already detected in surface waters, seawaters, groundwater, and some drinking waters (Kummerer 2000; Meade-Callahan 2007).

The fact that most pharmaceuticals are usually lipophilic and have often low biodegradability further indicates that they have a great potential of bioaccumulation and persistence in the environment (Christensen 1998). Therefore, aquatic organisms may be exposed to these drugs throughout their life cycles (Fent et al. 2006).

Despite some of these compounds showing low environmental acute effects, chronic toxicity and potential subtle effects are only marginally known (Fent et al. 2006).

Studies have shown that 17 β -estradiol (E₂) and estrone are responsible for most of the estrogenic activity in effluents of WWT (Solé et al. 2003), and these compounds have been found in natural waters, soil, and organic mud in several parts of the world (Belfroid et al. 1999; López de Alda and Barceló 2001; Ternes et al. 1999).

There are few studies on 17 β -estradiol toxicity, though this compound is being widely used as an oral contraceptive and as hormone replacement (Christensen 1998). 17 β -Estradiol is one of the most powerful xenoestrogens being considered an endocrine disruptor (Dorabawila and Gupta 2005), which may interfere both human and wildlife endocrine systems (Ankley et al. 1998; Birkett and Lester 2003; Ghiselli and Jardim 2007; Hughes 2007; Lintelmann et al. 2003).

Steroidal hormones show their effects on the target tissue by interacting with intracellular receptors and influencing the transcription of specific genes, leading to an increased or inhibited biosynthesis of specific proteins (Gillesby and Zacharewski 1998, Lintelmann et al. 2003). Biomarkers may be used for environmental monitoring, reflecting the interaction between a biological system and an environmental agent, which may be chemical, physical, or biological

(WHO 1993). Aquatic organisms may directly accumulate pollutants from water or through the ingestion of other contaminated aquatic organisms, the last process known as biomagnification. Thus, genotoxic pollutants may lead to the contamination, not only of specific organisms, but they may enter ecosystems and the human body through the food chain (Hoshina et al. 2008; Matsumoto et al. 2006). Sánchez-Galán et al. (Sánchez-Galán et al. 1998) demonstrated fish as good bioindicators, detecting contamination by genotoxic and mutagenic substances. Peripheral blood erythrocytes of these animals are frequently used to the micronucleus (MN) evaluation together with comet assay. These methods have demonstrated the feasibility of using fish as bioindicators for environmental assessment of genotoxic contaminants (Belpaeme et al. 1996; Buschini et al. 2004; Da Silva et al. 2002; Russo et al. 2004).

The study reported herein aimed to evaluate the genotoxic damage in erythrocytes of *Oreochromis niloticus* exposed to 6 ng/L 17 β -estradiol, using MN test, nuclear abnormalities (NA), and comet assays as genotoxicity endpoints.

2 Materials and Methods

2.1 Chemical

17 β -Estradiol was purchased from Galena (CAS 979-32-B, China). All the other chemicals were of analytical grade obtained from Sigma-Aldrich (Germany) and E. Merck-Darmstadt (Germany).

2.2 Fish

All the experiments were carried out with juvenile Tilapia (*O. niloticus*) species purchased from a fish farm in Bocaiuva do Sul, Paraná, Brazil. Fish measuring 13.5 cm were transported in aerated water and acclimated to laboratory conditions for 2 weeks prior to experimentation. During acclimatization and experimental periods, the fish were kept in 52-L aquaria, at 22°C in aerated water, with a pH value of 7 and under a natural photoperiod.

2.3 Experimental Design

The fish were exposed to water (control group—C) and water diluted E₂ at 6 ng/L (treated group—T).

The appropriate amount of E₂ was previously dissolved in aqueous ethanol, and this alcoholic solution was then added to 1 L of water from the aquaria to reach the previous initial nominal concentration.

The experiments were carried out with exposure periods of 24 h, 48 h, and 10 days, using test groups and control groups, each one with eight fish ($n=8$). The fish were sacrificed by spinal section after anesthetization with benzocaine 1% in water diluted (WD) exposure. Blood samples were collected from the spinal section.

2.4 Micronuclei and Nuclear Abnormalities Assessments

The MN test was performed according to Heddle (Heddle 1973) and to Schmid (Schmid 1975), and NA were evaluated according to Carrasco et al. (Carrasco et al. 1990) with modification.

Blood smears were prepared immediately after sampling, fixed with absolute methanol for 10 min, stained with 5% Giemsa dissolved in phosphate buffer (KH₂PO₄:Na₂HPO₄, 1:1, pH 6.8) for 7 min. For each fish, 2,000 erythrocytes were scored at 1,000× magnification.

Micronuclei have the following patterns: no connection with the main nucleus, the same color and intensity of the main nucleus, and sizes with less than one third of the main nucleus size (Moron et al. 2006).

The nuclear abnormality identification followed Carrasco et al. (Carrasco et al. 1990), according to four categories (blebbed, vacuolated, lobed, and notched). Blebbed nuclei show a relatively small evagination of the nuclear envelope, which seems to contain euchromatin. Vacuolated nuclei are those presenting vacuoles in a nucleus that does not contain nuclear material. Lobed nuclei are those presenting larger evaginations than the blebbed nuclei. A notched nucleus has an appreciable depth, but absence of nuclear material.

2.5 Comet Assay Procedure

The single-cell gel electrophoresis (SCGE)/comet assay was carried out according to (Singh et al. 1988) with modification.

The blood was obtained from fish as previously described and 12 µL aliquots diluted in 1,000 µL of PBS buffer (KCl+KH₂PO₄+NaCl+Na₂HPO₄). Microscope slides were first coated with 1.5% (w/v) high

melting point agarose and then coated with 120 µL of 0.5% (w/v) low melting point agarose at 37°C containing 10 µL of the diluted blood. The slides were placed into the lysis buffer (1 mL of Triton X-100, 10 mL of DMSO, and 89 mL of pH 10 lysis solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 8 g of NaOH, and 10 mL of 1% (w/v) sodium lauryl sarcosinate in 890 mL of ultrapure water) for 1 h, at 4°C. After lysis, the slides were incubated in 300 mM NaOH+1 mM EDTA buffer (pH>13) for 20 min to denature the DNA and then submitted to electrophoresis at 32 V and ~300 mA for 30 min, in a horizontal gel electrophoresis chamber. The slides were then neutralized with 0.4 M Tris for 5 min (three times) and fixed in 100% ethanol for 5 min. For each fish, 100 nuclei were analyzed. The slides were stained with ethidium bromide (0.2 mg mL⁻¹) and analyzed under a Nikon fluorescence microscope equipped with a B-3^A filter (excitation: $\lambda=420$ –490 nm, emission barrier: $\lambda=520$ nm) and 40× objective lens. The nuclear material was visually classified according to the fragment migration as undamaged (class 0), slightly damaged (class 1), moderately damaged (class 2), medium damaged (class 3), and highly damaged (class 4) (Collins 2004).

2.6 Statistical Analysis

Normally distributed samples were tested with parametric test (*t* test and ANOVA), and non-normally distributed ones with non-parametric tests (Mann–Whitney and Kruskal–Wallis tests). The results were considered statistically significant when $P\leq 0.05$. Comparisons were carried out with negative controls, for different exposure periods (24 h, 48 h, and 10 days). All analyses were performed using the SPSS 12.

3 Results

3.1 Micronucleus Test

Data on MN frequency of fish erythrocytes are shown in Table 1. There was a significant difference between control group and treated group (Mann–Whitney $P=0.036$) for 10-day exposure to 6 ng/L 17 β -estradiol. No significant difference (Mann–Whitney $P=0.060$) was observed for 24-h and for 48-h exposures (Mann–Whitney $P=0.207$) using MN test.

Table 1 *P* values for evaluations of 6 ng/L E2 genotoxicity in erythrocytes of *O. niloticus*

Assay	Exposure Time	Average	Value of <i>P</i>	Value per experiment						
				1		2		3		
				Average	<i>P</i>	Average	<i>P</i>	Average	<i>P</i>	
Micronucleus quantity	24 h	C	4.31	0.060	44.29	0.081	54.50	0.099	104.5	0.791
		T	12.5		89.38		114.0		147.9	
	48 h	C	3.31	0.207	178.1	0.560	31.88	0.162	107.2	0.788
		T	4.38		133.6		75.50		179.6	
	10 day	C	4.79	<i>0.036</i>	69.88	0.386	41.00	0.184	126.1	0.137
		T	2.54		32.88		96.88		84.50	
Nuclear abnormality quantity	24 h	C	67.66	<i>0.018</i>	4.43	<i>0.049</i>	3.13	<i>0.012</i>	5.38	0.753
		T	116.1		24.1		5.38		8.13	
	48 h	C	–	–	6.29	0.418	1.63	<i>0.026</i>	2.00	<i>0.036</i>
		T	–		6.00		3.88		3.25	
	10 day	C	78.9	0.703	4.88	0.833	3.50	0.073	6.00	0.753
		T	71.42		2.50		2.13		3.00	
Comet scores	24 h	C	–	0.227	229.43	0.056	299.25	0.105	279.75	0.093
		T	309.9		303.13		327.00		299.50	
	48 h	C	–	<i><0.001</i>	28.71	<i>0.002</i>	305.5	0.172	239.88	<i>0.021</i>
		T	270.9		267.86		272.13		272.88	
	10 day	C	–	0.105	249.63	<i>0.002</i>	131.13	0.318	274.13	0.151
		T	–		329.63		108.13		205.88	

C control group, *T* treated group, – not normal distribution

Data written in italics corresponded to significant differences

3.2 Nuclear Abnormality Evaluation

Other NA of fish erythrocytes were also evaluated, and there was a significant difference between control and exposed groups (Mann–Whitney $P=0.018$) for 24 h of 6 ng/L 17 β -estradiol exposure, but no significant difference (Mann–Whitney $P=0.227$) for 48-h and (Mann–Whitney $P=0.703$) for 10-day exposures, as shown in Table 1.

Comparison of treatments based on each category of NA indicates there was a significant difference between control and exposed groups only to the cell number of notched nuclei (Mann–Whitney $P=0.024$) at 24-h exposure and of lobed nuclei (Mann–Whitney $P=0.003$) at 48-h exposure. However, there were no significant differences of lobed (Mann–Whitney $P=0.483$), blebbed (Mann–Whitney $P=0.577$), vacuolated (Mann–Whitney $P=0.088$), and notched nuclei (Mann–Whitney idem) for 10-day exposure.

3.3 Comet Assay

Comet scores of treated groups and control groups are shown in Table 1. There was a significant difference between control and exposed groups (Mann–Whitney $P<0.001$) only for 48-h exposures to 6 ng/L 17 β -estradiol. No significant differences (Mann–Whitney $P=0.227$) were observed for 24-h and for 10-day exposures (Mann–Whitney $P=0.105$).

Significant differences were identified between control and 24-h exposure groups in relation to the cell number of class 1 and class 2 fragment migration (Mann–Whitney $P=0.005$ and $P=0.013$, respectively), the same being observed for cell number of fragment migration class 2 (Mann–Whitney $P=0.036$) of 10-day exposure. No significant difference was registered to cell number of any fragment migration at 48-h exposure.

4 Discussion

The herein study contributes significantly to the knowledge of E₂ ecological consequences in the aquatic environment.

The determination of MN rates has been frequently used to evaluate structural and numerical chromosomal aberrations induced by clastogenic and aneugenic agents (Grisolia and Starling 2001; Heddle et al. 1991; Ventura et al. 2008). Other papers have described NA analysis (Ayllon and Garcia-Vazquez 2000; Çavas and Ergene-Gozukara 2003), as well as comet assay (Frenzilli et al. 2009; Ventura et al. 2008), complementing MN test in cells of fish exposed to genotoxic substances.

Results for 6 ng/L E₂ exposure of *O. niloticus* for 24 and 48 h showed that these erythrocyte tests may be effective in detecting E₂-induced genotoxicity and showed the prolonged and extensive use of these drugs as dangerous. These results are in accordance with those previously reported by Hundal et al. (Hundal et al. 1997), for *Ames Salmonellar S9* exposed to 1, 10, and 100 mg mL⁻¹ of estrogen for a period ranging from 24 to 48 h, which discussed the estrogen potential to promote carcinogenesis, to inhibit the synthesis of DNA or cell proliferation, or to form DNA adducts.

Although there have not been significant differences between the control group and the treated group for the period of 24 h and of 48 h on the MN frequencies, exposure to E₂ for a period of 10 days showed a decrease in the MN frequency when compared to the negative control group. Although the literature has reported the MN test as a good biomarker in fish (Guilherme et al. 2007, Strunjak-Perovic et al. 2008), this result may be influenced by the erythrocyte renewal kinetics, since studies have shown that the MN frequency in erythrocytes of fish tends to decline versus chronic exposure to pollutants (Campana et al. 1999; Çavas and Ergene-Gozukara 2005b).

The formation of MN in cells that are in division is the result of a chromosomal break (great damage) because of failure to repair or incomplete repair of the lesion in DNA, or because the chromosome segregation does not work appropriately in the process of mitosis (Bonassi et al. 2007), so it could be inferred that a significant difference in the amount of erythrocyte MN for exposure period of 10 days could

be caused by the activation of repairing mechanisms in the *O. niloticus* at chronic exposure to E₂. Additional studies are required for further information on this mechanism.

Although a significant increase in erythrocyte micronuclei in fish after 6 ng/L E₂ 24-h exposure has not been recorded, studies with human cells have demonstrated that E₂ and estrone induce an increase in the number of micronuclei cells in a concentration of nanograms per liter (Yared et al. 2002).

These variations may also be a direct consequence of the sensitivity behavior and the niche of the species, as the number of MN in the cells of fish may be variable. Some studies have reported natural differences in the number of MN in some species (Gustavino et al. 2001), mainly because organisms may differently respond to stress due to transcription of specific genes, or different levels of absorption and metabolism of genotoxic agents, DNA repair, cell death, control of cell cycle, and immune response (Goode et al. 2002).

Many chemical compounds may simultaneously induce the formation of both MN and other NA, or they may cause only one of these changes (Carrasco et al. 1990; Pacheco and Santos 1998). This may justify a significant difference only observed for the E₂ exposure period of 24 h when assessing NA and not for the MN Test and the comet assay.

Studies with fish *Dicentrarchus labrax* L. exposed to E₂ at higher concentrations (200 and 2,000 ng/L), both in intraperitoneal dose, for 10 days showed genotoxic effects assessed by increased NA frequencies of erythrocytes (MN and other NA analyses) in comparison with the control group (Teles et al. 2006). The authors showed that the genotoxic changes observed, depending on the environmental doses tested for them, were more significant when compared with intraperitoneal administration.

The environmental genotoxic potential for 6 ng/L E₂ may be explained due to the fact that it may connect to DNA and to proteins inducing a simple break mechanism in the DNA and the MN formation (Yared et al. 2002).

The herein study showed an increase in the frequency of cells with notched nuclei after 24-h exposure to E₂ and of cells with lobed nuclei after 48-h exposure. Similar results in the number of cells with lobed nuclei

have been previously reported (Çavas and Ergene-Gozukara 2005a) for the species *O. niloticus* exposed to effluents from a petroleum refinery and a chromium processing plant.

The fact that there was no significant difference between the treated group and the control group for the exposure periods of 24 h and 10 days in the comet assay may be due to individual variations in the ability to repair DNA (Schmezer et al. 2001). This may be a quick process, with half life of only a few minutes to hours, the latter in case of repair by the oxidation of DNA bases (Collins and Horvathova 2001). It is possible to suggest that incomplete DNA condensation did not occur in these periods of exposure (Koppen et al. 1999, Lemos et al. 2005). Fragmentation of DNA ribbon may be considered a type of potentially pre-mutagenic lesion (Kammann et al. 2001), and breaks in the DNA ribbon may be related to the mutagenic and carcinogenic properties of chemical compounds, as observed by some authors (Frenzilli et al. 2000; Matsumoto et al. 2006).

E₂ genotoxicity may be also justified because estrogenic hormones are generally excreted in their conjugated inactive organic forms (Desbrow et al. 1998). The conjugated form of these hormones is easily hydrolyzed, returning to the estrogen active form, due to the action of bacteria that produce β-glucuronidase or sulfatase enzymes (Desbrow et al. 1998; Ternes et al. 1999). The rate of conversion to estrone in the wastewater treatment is high, suggesting that this transformation is heavily favored (Desbrow et al. 1998; Ternes et al. 1999).

The estrogens are converted to catechols (hydroxylated estrones), and these result in quinones, which interact with DNA. The quinones, consequently, result in mutation through the redox cycle of oxidative damage in the DNA (Hundal et al. 1997; Joosten et al. 2004). The estrogens may even induce the formation of endogenous DNA adducts in humans and other animals (Liehr 1990), and they may also promote sister chromatid exchange (Joosten et al. 2004). This interaction may result from the fact that E₂ has a log K_{ow} (log *P*) of 3.94 (Colucci et al. 2001), which means high liposolubility and suggests its high capacity to bioaccumulate in lipid structures and then to generate effects in nuclear level.

Further studies on E₂ genotoxicity must therefore be carried out to monitor the rate of lesion removal and the kinetics of the process.

The herein study suggests the feasibility of using MN and NA assays as a rapid assessment of E₂ environmental influence on *O. niloticus*. Although the comet assay was a good indication for the evaluation of 48-h exposure effects, it is a more complex technique in both financial and technical aspects.

5 Conclusions

17β-Estradiol (6 ng/L) gave rise to disturbances in blood cells of *O. niloticus*. This compound may be considered a clastogenic chemical contaminant for 24-h, 48-h, and 10-day exposures. The combined results showed that acute and chronic exposures to 17β-estradiol produce mutagenic and genotoxic effects on the blood cell of *O. niloticus*. The combination of MN, NA, and comet assays proved to be suitable and useful in the evaluation of the 17β-estradiol genotoxicity.

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Conflict of Interest Statement The authors declare that there are no conflicts of interest.

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