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Reproductive expression dynamics and comparative toxicological perspective of beta estrogen receptor gene in the male wall lizard, *Podarcis sicula* Rafinesque, 1810 (Chordata: Reptilia)

G. GUERRIERO1,2*, R. DI GIAIMO1*, O. HENTATT3, F. KH. ABDEL-GAWAD1,4, S. TROCCHIA1, D. RABBITO1, & G. CIARCIA1,2

1Department of Biology, University of Naples Federico II, Naples, Italy, 2Interdepartmental Research Center for Environment (I.R.C.Env.), University of Naples Federico II, Naples, Italy, 3Department of Biotechnology and Health, Institut Supérieur de Biotechnologie de Sfax, Sfax, Tunisia, and 4Centre of Excellence for Advanced Sciences (CEAS), National Research Centre, Giza, Egypt

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Abstract

Over the last few decades, due to its relevant function in male reproduction assessment, important molecular achievements have been made in the molecular characterization of estrogen receptor genes in various species. Our work focuses on a male seasonal breeder, the bioindicator *Podarcis sicula*, because of its peculiar gonadal anatomy, similar to that of humans. Based on the cloned lizard’s gene sequence fragment of estrogen receptor beta, *esr2* (GenBank JN705543.1), we found DNA binding domain identity of 99% as well as a homologous sequence with humans. Furthermore, in order to better illustrate how this gene is regulated in the lizard’s reproductive system organs, we investigated the transcriptional activity of *esr2* in brain and testis tissues during mating and winter stasis phases of the reproductive cycle. Quantitative real time-polymerase chain reaction (qRT-PCR) analyses performed on male gonadal tissues demonstrate a significant increase in *esr2* expression during mating compared to the winter stasis period, while in the brain, *esr2* shows the opposite trend. Next, we provide morphological evidence of the detrimental effect on spermatogenesis of a pure anti-estrogen treatment (ICI 182,780) and the corresponding effect on *esr2* expression in lizard specimens during the mating period which, upon treatment, was found to be no different from the expression levels in winter stasis both in the brain and in the testis. In this study, we explore the potential use of *Podarcis sicula* as a model for human testis development and maturation, as well as *esr2* expression for toxicological screening in one-testis gonadectomy.

Keywords: esr2 phylogenetic similarity, Podarcis sicula, qRT-PCR, ICI 182, 780, toxicological assessment

Introduction

Estrogen receptors, like all members of the nuclear receptor superfamily, share a common domain organization consisting of a region located in the central part of the gene that binds DNA (DNA binding domain, DBD) which is highly conserved; a domain involved in binding with the ligand, in the dimerization and transactivation (ligand binding domain, LBD); variable N-terminal domain and C-terminal regions; and a hinge region between the DBD and LBD showing phylogenetic steroid receptor conservation (Gronemeyer & Laudet 1995; Mahfouz et al. 2016). Estrogen receptor transcriptional activity is induced by estrogens in response to changing demands due to factors such as female and male reproductive status, cognitive function, mood and environmental pollution (Zhang et al. 2009; Tohyama et al. 2016; Cooke et al. 2017). In previous work in this field, estrogen receptor alpha (ER-ALFA, *esr1*) and estrogen receptor beta (ER-BETA, *esr2*) were immunodetected, and their cDNA was cloned and quantified in many vertebrates (Katsu et al. 2004; Verderame & Limatola 2010; Zhang et al. 2017). Further, in teleost fish a third estrogen receptor class was identified and...
named gamma (ER-GAMMA) (Hawkins et al. 2000), while in some sharks the presence of only one estrogen receptor form was found (Katsu et al. 2010). All cloned receptors responded similarly to estrogen level variations whether with down- or up-regulation of expression in relation to tissue, gender, and the season for each species. However, they responded differently to estrogenic endocrine disruptors (Greytak & Callard 2007; Tohyama et al. 2016). Signal transduction through these nuclear receptors leads to dramatic changes in gene expression programs in different vertebrate cell types, typically due to their binding to DNA or to transcription modulators (Guerriero & Ciarcia 2001; Guerriero 2009; Guerriero et al. 2018b). *esr1* and *esr2*, in particular, exhibit different biological roles and functions, as demonstrated by knock-out experiments in mice (Blatter & Mahoney 2015). The proposed estrogen receptor functions of *esr2*, in particular, include anti-proliferative action, regulation of apoptosis, control of antioxidant gene expression, modulation of immune responses and DNA repair (Nikolos et al. 2018). Furthermore, *esr2* is already known to be a key element in the signal transduction pathway for endocrine disruptor compounds (Tohyama et al. 2016). Xenobiotics, as well as substances able to compete with estrogens in the binding with *esr2*, even if they were usually unable to activate it, are known as anti-estrogens (Greytak & Callard 2007; Verderame et al. 2014; Tohyama et al. 2016; Walker & Gore 2017). Some of the most used anti-estrogens in cancer therapy are tamoxifen (Nolvadex) and raloxifene (Evista), which act as agonists or antagonists, depending on the target cells (Zhang et al. 2007; Arevalo et al. 2011; Jensen et al. 2018). Another known anti-estrogen is the ICI 182,780 (trade names Faslodex, Fulvestrant), which only competes with estrogens in the binding with ERs, depending on the target cells (Zhang et al. 2007; Arevalo et al. 2011; Jensen et al. 2018). Another known anti-estrogen is the ICI 182,780 (trade names Faslodex, Fulvestrant), which only exhibits anti-agonistic action toward estrogens and is thus considered a pure anti-estrogen (Alfinito et al. 2008; Guerriero et al. 2012; Gao et al. 2016). It is able to cross the blood-brain barrier, penetrate into brain and hypothalamic tissues, and affect known neuroendocrine functions. The *esr2* gene has been studied in different tissues in reptiles including brain and gonads (Katsu et al. 2004, 2010; Verderame et al. 2014; Mahfouz et al. 2016), but its neuronal expression has never been investigated in relation to reproductive events although its importance in the regulation of reproduction is unquestionable. The *esr2* gene coding for ER-BETA has been cloned and characterized in many fishes, birds, amphibians and reptiles (Katsu et al. 2004, 2010; Katsutoshi et al. 2007; Verderame & Limatola 2010; Zhang et al. 2014, 2017). The study of its expression in the brain may allow the monitoring of species. Specifically, the expression of *esr2* can be used as a biomarker and its assessment may be considered a useful component of strategies for the conservation of species at risk of extinction (Guerriero et al. 2018b). This is because gametogenesis is under the control of the hypothalamic-pituitary system, which is substantially similar among the various vertebrate classes (for a review see Guerriero 2009), and their hormones. Sex steroid hormones, along with the pituitary gonadotropins, are able to modulate reproductive events, thanks to the presence of specific nuclear receptors and microRNAs (Zhang et al. 2009; Cao et al. 2018). The importance of the linkage between these structures is evident especially in seasonal breeding species, where environmental stimuli are integrated at the level of the central nervous system with the internal hormonal system and with endogenous antioxidants to trigger reproductive behavior (Guerriero et al. 2003, 2004, 2012, 2018b; Guerriero 2007). The use of seasonal breeding animals allows analysis of the physiological changes of the gonad and the monitoring of possible molecular “pathways” of the brain-gonad axis that regulate the progression of events for spermatozoon and oocyte formation (Guerriero 2009; Guerriero et al. 2018b). Previous research in our laboratories has shown that in the lizard *Podarcis siculus* (*Podarcis sicula*) testis, the estrogen receptor protein is at the highest levels during winter stasis, whereas the lowest gonadal levels occur during maximum activity or mating (Ciarcia 1993). The data suggest a seasonal modulation by the brain along the hypothalamic-hypophysis-gonad axis. For instance, results of testis estrogen measurements demonstrated more elevated levels during the mating phase. These results support the fact that the highest amount of local binding of estrogen occurs in winter stasis whereas estrogen plasma levels are higher in the mating phase (Andò et al. 1992). Evidence has been found in *Podarcis* testis demonstrating the direct influence of estrogens on the function and maturation of germ cells (Chiefi & Varriale 2004). Recently, our laboratories reported the *Podarcis sicula* *esr2* sequence (GenBank JN705543.1). In the current study we report the characterization and expression of the *esr2* dynamic in the brain and gonad of the male lizard. We describe the bioindicator *Podarcis sicula* followed through a decade of study, and the possibility of employing it, from a comparative toxico-physiologic perspective, as phylogenetically close to mammals, having similar anatomical germinal cell compartments. Finally, to expand our understanding of the role of *Podarcis sicula* as a bio-sentinel we address ICI 182,780 treatment to identify in the brain, as well as in the testis, its toxicological effect on germinal
cells. These predictable answers will help us both to understand the brain-gonad feedback in normal status, with a possible role of *Podarcis sicula* testis gonadectomy, and to identify variability within the established brain-gonad dynamics when there is interference by anti-estrogen compounds.

**Materials and methods**

**Wall lizard maintenance and experimental design**

Sexually mature male wall lizards (total number 200 with n = 10 per each seasonal collection examined/10 years, weight range 10–14 g) identified as *Podarcis sicula* were collected (wild-caught) in the reference site of Monti di Lauro, Avellino (I), during two periods of the year: mating period, when there is full spermatogenetic activity (May), and winter stasis when the spermatogenesis is quiescent (November), in the period 2008–2017. The lizards were captured and the experiments were carried out in accordance with the ethical provisions enforced by the National Committee of the Italian Ministry of Health on in vivo experimentation (Department for Veterinary Public Health, Nutrition and Food Safety, SCN/2D/2000/9213). For 10 days before the experiments, the captured animals were housed in large terraria containing rocks, sticks and water. The animals were fed with mealworms *Tenebrio molitor* and fresh fruit ad libitum. All efforts were made to minimize animal suffering and to reduce the number of specimens used. Since they are heterotherms, they were euthanized at 4°C. For each specimen, brain and gonad were aseptically sampled. Some testes were dissected out and weighed, and only one plunged in Steve’s solution with (n = 2) and without treatment with ICI 182,780 (n = 2), whereas the others, together with other tissues and organs, were quickly frozen at −80°C for molecular analysis.

**Treatment with the estrogen receptor down-regulator ICI 182,780**

The treatment took place during the mating period (May). Animals (n = 5 of mating collection/10 years) were subjected to a thermo-photoperiodic regime typical of the winter stasis period with a 16:8 h dark:light cycle and exposed to a controlled temperature of 10°C±1.5°C. Specimens were injected every 24 h with a subcutaneous dose of anti-estrogen ICI 182,780 (Tocris, catalog number 1047; M.W. 606.77; 6 mg/0.25 mL almond oil/animal). In parallel, control animals (n = 2) were injected with almond oil only (0.25 mL/animal/day). Collection of tissues and organs from treated animals occurred on the 21st day after the beginning of treatment, with three animals in each of the years from 2008–2017.

**Histological analysis of the testis**

Only one testis of three euthanized lizards for each condition (mating with and without ICI 182,780, and stasis) was fixed in Steve’s solution and subsequently embedded in paraflin-celloidin according to Petefi (Mazzi 1977). Morphological staining was carried out on histological sections (7 μm thick) using Galgano’s trichome, and histological analysis was done using a Nikon-MicroPhot-FXA light microscope.

**RNA extraction and cDNA synthesis**

Total RNA was extracted by homogenization tissue in Trizol (Invitrogen), according to the manufacturer’s instructions. Briefly, testes and brain (50 mg) from euthanized *Podarcis sicula* (n = 5) of each phase of the reproductive cycle – mating and winter stasis – and in mating treated with ICI 182,780, during the years 2008–2017, were homogenized in 1 mL of Trizol reagent buffer, and then the homogenized samples were kept at room temperature for 15 min. A volume of 0.2 mL of chloroform per 1 mL of Trizol reagent was added. The samples were vortexed vigorously for 15 s, then incubated for 3 min at room temperature and centrifuged for 15 min at 4°C at 12,000 × g. After centrifugation, the upper aqueous layer was transferred to a fresh tube, then RNA was precipitated after mixing with isopropyl alcohol. Isopropyl alcohol (0.5 mL) was added to 1 mL of Trizol reagent used in the initial homogenization. Afterward, samples were incubated for 10 min at 30°C and centrifuged at 4°C for 10 min at 12,000 × g. The RNA pellet was detected after removing supernatant, then washed with 1 mL of 75% ethanol, centrifuged at 7500 × g for 5 min at 4°C. The RNA pellet was air-dried for 10 min, then resuspended in 100 μL diethylpyrocarbonate (DEPC)-treated water and stored at −80°C. The quality and the amount of purified RNA were assessed using spectrophotometer measurements at 260 and 280 nm (only samples with a ratio 260/280 ≥ 1.8 were accepted and further processed) and by electrophoresis under denaturing conditions on 1% agarose gel according to Raven et al. (1979). To avoid the amplification of contaminant genomic DNA, we purified the total RNA from genomic DNA with an Ambion RNA-free kit. Total cDNA was synthesized from total RNA in duplicates for each sample to minimize reaction variations. One hundred nanograms of RNA in 20 μL reaction volume was used for each cDNA synthesis reaction using
MMLV reverse transcriptase (Promega ImpProm II kit) according to the manufacturer’s instructions.

**DNA sequence alignment and phylogenetic tree**

The partial estrogen receptor beta (ER-BETA) protein sequence from *Podarcis sicula* (AFD18855.1) was aligned to all of the reference proteins available in the National Center for Biotechnology Information (NCBI) database by using protein-protein BLAST (BLASTp). Identified sequences were aligned and used to build a phylogenetic tree by using COBALT.

**Quantitative real time-polymerase chain reaction (qRT-PCR)**

Quantification of *esr2* mRNA by RT-PCR was performed on five *Podarcis sicula* of each examined condition and the analyses were performed on two different tissues: testis and brain. 12S rRNA was used as a reference gene. qRT-PCR was carried out in a OneStep Plus Real-Time PCR System (Applied Biosystems), using SYBR Green (Applied Biosystems). Each reaction was performed in a 20-μL reaction volume containing 1 μL of a 1:5 dilution of cDNA preparation, 10 μL of 2x SYBR Green qPCR Kit (Applied Biosystems), and 5 μL 0.8 μM of each primer (PRIMM Biotech Products and Services, Milan, Italy). The specificity of the qRT-PCR was verified by sequencing the reaction product. Negative controls were performed on samples without cDNA in the reaction mix. Melting curves from each reaction were analyzed and all showed only one peak at the same Tm, indicating the absence of primer dimers or side products. The primers were designed on the *esr2* sequence (JN705543.1) using the Primer3 software. The forward primer was 5’-AAGAGAGCCGCTGTGGCTATC-3’ and the reverse primer was 5’-CAGTGCAATTGCACAGCAGTT-3’, giving a product size of 150 base pairs (bp). The PCR for the genes included an initial denaturation step at 95°C for 5 min, followed by 45 amplification cycles consisting of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and an extension at 72°C for 60 s, plus a final extension at 72°C for 1 min. To ensure that a single product was amplified, melt curve analysis was performed on the PCR products at the end of each run. The PCR products were further assayed on a 2% agarose gel. The relative expression value was calculated as a fold change with the formula 2- ΔΔCt (Livak & Schmittgen 2001).

**Statistical analysis**

Multiple group data were analyzed using one-way analysis of variance (ANOVA). The Student’s *t*-test was used to compare means between the groups. Statistically, significant differences are defined outside of the 95% confidence interval. Data are shown as a mean ± standard error of the mean (SEM). Statistical parameters are reported in the respective figures and figure legends.

**Results**

**DNA sequence alignments**

Figure 1a shows the sequence alignment of *Podarcis sicula* protein with the four best protein matches in the BLAST database of other species that belong to different families of the order Squamata, class Reptilia – *Phytolacca emodi* (Pythiornidae), *Plestiodon finitimus* (Scincidae), *Anolis carolinensis* (Polychrotidae) and *Elaphe quadrivirgata* (Colubridae) – together with the human sequence showing phylogenetic relationships based on multiple sequence alignment and conserved protein domains. Figure 1b displays identities (%) with *Podarcis sicula* partial sequence (SmartBLAST) as well as the conserved domains of *esr2*. In particular, *Podarcis sicula* DBD shows 99% sequence identity with the human sequence. The LBD is also highly conserved between *Podarcis sicula* and *Homo sapiens*, showing 83% sequence identity.

Figure 1c shows the detailed multiple sequence alignment of the selected proteins, and the DBD and LBD are indicated.

**Expression levels of esr2 during the reproductive stages**

Figure 2a depicts the typical testicular organization during the mating period. The seminiferous tubules are well developed and all the germinal cells in different stages of maturation are clearly evident, including many sperm. Figure 2b shows representative sections of testis during the winter stasis phase; in the testis, the spermatogenetic processes appear substantially slowed down, with a reduced germinal epithelium, large lumen and gaps between the germ cells. Many degenerations are found in the seminiferous tubules, especially at spermatids; only a few very rare sperm are present, which will not be used for fertilization. After confirming the reproductive phase, we investigated the expression of the *esr2* gene by qRT-PCR both in the testis and in the brain (Figure 2c). We included
Figure 1. (a) Phylogenetic tree of Podarcis sicula ER-BETA partial protein sequence and the four best sequence matches together with the human sequence, showing phylogenetic relationships based on multiple sequence alignment and conserved protein domains. (b) SmartBLAST identities (%) with Podarcis sicula partial sequence are indicated. The values 99% and 83% are the sequence identities of human DBD and LBD domains, respectively, vs Podarcis sicula. (c) Multiple sequence alignment of the sequences aligned in (a). The red color indicates identical residues (Cobalt). Conserved domains are indicated: DBD in blue and LBD in green.
brains in our analysis to assess the possible impact of esr2 on the crosstalk brain-gonadal axis. Finally, we merged the data obtained in the various years considered to improve statistical power in the analysis of the combined pool of data. The qRT-PCR analysis in the gonadal tissues showed a significant increase of estrogen receptor beta gene expression during the mating phase compared to the winter stasis phase. Differently from gonads, the expression of esr2 in the brain is significantly higher in the winter stasis phase compared to the reproductive phase.

We also analyzed the expression levels of esr2 upon treatment with the estrogen receptor down-regulator ICI 182,780, both in the brain (Figure 3b) and in male gonads (Figure 3c) of lizards collected in the period 2008–2017. The anti-estrogen inhibits the expression of esr2 in the brain, showing levels not statistically different from those in the winter stasis period, in line with the histological results. Along the same line, in the male gonads, treatment with ICI 182,780 is shown to be effective as evidenced by expression levels in the treated animals being lower than in animals in the mating period, but injected with vehicle only; and these lower levels were also not significantly different from those of the winter stasis period.

Discussion
In this study, we first analyzed the partial sequence of the mRNA for esr2, which we identified in the lizard Podarcis sicula (deposited under the accession number GenBank JN705543.1) looking for sequence homologies and confirming the evolutionarily well-conserved domains. Observing in detail the amino acid sequence of esr2 of Podarcis sicula, we clearly recognize the three classical domains of the estrogen receptor superfamily (Guerriero et al. 2009; Mahfouz et al. 2016). Estimating the evolutionary distance between the partial sequence of esr2 of the Podarcis sicula lizard and the sequences of esr2 deposited in the BLAST database, it emerges, as expected, that

Treatment with an estrogen receptor down-regulator (ICI 182,780)
In order to better understand the role of esr2 in the regulation of gonadal activity, we treated animals with the anti-estrogen ICI 182,780. Figure 3a highlights the histological organization of gonadal tissue upon treatment with the anti-estrogen. Treatment of animals with estrogen receptor down-regulator during the mating period of the reproductive cycle induces histological changes. In the testis, some seminiferous tubules show a slowdown of spermatogenesis processes, with a reduced lumen and few sperm. Severe testicular changes occurred, including an arrest of spermatogenesis at the secondary spermatocyte level. Treatment with ICI 182,780 induces a reorganization of the seminiferous tubules comparable to the winter stasis period.
the sequence esr2 of *Podarcis sicula* is placed among the reptiles. The sequence alignments also indicated that the lizard sequence is close to the birds’ orthologs but, interestingly, closer to the mammals than to amphibians and fishes (data not shown). In particular, an overall 76%
sequence identity between human and lizard esr2 sequences emerged. It is interesting, then, to emphasize how the conserved DNA-binding domain shows an identity of 99% with Homo sapiens as well as to the closest species, clearly indicating a strict correlation of structure/function in DNA binding and recognition of target genes.

Furthermore, we demonstrate that Podarcis sicula estrogen receptor is actively transcribed in the brain as in another endocrine tissue already reported, the testis (Ciarcia 1993; Verderame et al. 2014). The lowest gene expression levels were detected in the brain with the concomitantly highest esr2 transcription levels in testis during mating. Moreover, comparison of esr2 gene expression during the mating period compared to the winter stasis of the reproductive phase showed upregulation in the testis and downregulation in the brain. Those seasonal variations in gene expression followed the estradiol pattern in Podarcis sicula testis (Ciarcia 1993), and this result is in agreement with various studies showing that testes produce estradiol and that esr2 is very sensitive to estradiol changes as, for example, in mammal rat testis, where ER-BETA is detected in spermatogenetic cells of various stages and in Sertoli cells, suggesting that estrogens directly affect germ and somatic cells during testicular development and spermatogenesis (Oliveira et al. 2001; Cooke et al. 2017). Podarcis is one bioindicator species for estradiol exposure altering esr2 expression as detected by in situ hybridization studies by Verderame et al. (2014), and estrogen receptor mRNA using real-time PCR in primary cultures of lizard testis cells (Cardone et al. 1998). Our laboratories have demonstrated that there are close dynamics between brain and testis and that seasonal correspondence in the main phases of the reproductive cycle follows the feedback of the brain-gonad axis. Podarcis sicula, as a seasonal breeder, allowed us to better characterize how the gene esr2 is regulated in the brain of this lizard.

In order to study the expression levels of esr2 from Podarcis sicula, we investigated, by qRT-PCR, the esr2 expression in both the gonads and the brain of adult male lizards collected during the winter stasis and the mating period. The histological analysis of the tissues allowed verification of the expected tissue organization of the relative reproductive period and the effect of anti-estrogen treatments.

The analysis of the expression of esr2 in the reptile Podarcis sicula, by qRT-PCR, highlighted an interesting seasonal dynamic between the brain and the gonads, as in mammalian species (Pentikainen et al. 2000; Cooke et al. 2017). In the lizard, levels of estradiol show seasonal variations in accordance with the reproductive cycle via ER-BETA (Chieffi & Varriale 2004). The estrogen receptor levels are modulated by a series of complex processes that involve the control of expression and post-transcriptional modifications (Guerriero et al. 2005b, 2009; Greytak & Callard 2007; Tohyama et al. 2016; Cooke et al. 2017).

Our present and previous results indicate that the testis should be considered a valid tissue model for endocrine disruption assessment. This idea may be supported by the high conservation degree of the sequences of the DBD and LDB domains and by the spatial organization of germ cells. An interesting confirmation is provided by the data of Cohen et al. (2012) in the lizard Anolis, that show a similar neurodistribution in the brain between vertebrates as also confirmed by Guerriero et al. (2009) and Mahfouz et al. (2016). The work by Cohen et al. (2012) examines reptile brain ER-BETA distribution, documenting a similar pattern to that found in birds and mammals, which suggests that this receptor may perform similar functions across multiple vertebrate taxa, whereas lizard testis coexpresses androgen receptor and estrogen receptor alpha and beta (Verderame et al. 2014). Further studies are needed to elucidate the general vertebrate mechanism underlying esr2 actions and fertility, but there is strong evidence of ER-BETA predominance within the seminiferous tubules in the early gametogenesis event (O’Donnel et al. 2001).

In recent years, many high-persistence molecules, defined as emerging contaminants, have become of concern because of their ability to disrupt the endocrine system and their recalcitrance in the environmental matrix (Santos et al. 2010). Particular importance has been given to pharmaceutical products derived not only from human but also from veterinary use. Among the therapeutic classes, antibiotics, epileptic agents and sex hormones are predominant (Santos et al. 2010). Steroids such as the synthetic hormone 17 alpha estradiol (main hormone of the small contraceptive) and the natural hormone 17 beta estradiol (used as a drug in hormone replacement therapy) are added to the anti-steroids; in particular the antiestrogens, based on their mechanism of action, are classified into impeded antagonists, such as estradiol, which interacts with ER but rapidly dissociates; aromatase inhibitors, such as anastrozole, which block the conversion of androgens into estrogens; the triphenylethylene antagonists which in turn are divided into two families: the selective ER modulators, such as the drug Nolvadex, and the selective destroyers of ER, such as Faslodex whose bioactive molecule is ICI 182,780. Our attention has focused on ICI 182,780. It is known that estradiol administration stimulates proliferation of spermatogenesis in otherwise inactive testes in teleosts, amphibians and reptiles, and in each of these classes, tamoxifen
and ICI 182,780 prevent this stimulation process (Minucci et al. 1995; Guerriero et al. 2000; Chiefi et al. 2002). On the basis of the human species’ esr2 identity with Podarcis sicula, we treated the bioindicator Podarcis sicula with ICI 182,780. ICI 182,780 is a 7-alkylsulfonyl, an analog of endogenous estrogen 17-beta-estradiol. It binds to estrogen receptors with high affinity, preventing binding with estrogens. In this way it prevents the receptor’s dimerization and promotes its degradation, causing the abrogation of the transcription of the sensitive estrogen genes (Morris & Wakeling 2002). The damage caused by ICI 182,780 at the brain level is already known (Chiefi et al. 2002; Alfinito et al. 2008; Guerriero et al. 2012; Gao et al. 2016) and can be studied following the sensitivities of several different biomarkers and approaches (Guerriero et al. 2003, 2005a,b; Bartriromo et al. 2013; Guerriero et al. 2018a, 2018b; D’Errico et al. 2018). In particular, morphological studies have shown that ICI 182,780 changes the hypophysis histomorphometrically with an increase and/or reduction of its weight (Gao et al. 2016), and increases the concentration of calcium channels (Zhao et al. 2006). Biochemical studies have allowed detection of alterations of the lactate dehydrogenase pattern (Nunez & McCarthy 2003) and extracellular phosphorylation of the kinases ERK1/2 (Wong et al. 2003). Indeed, through molecular studies it was possible to demonstrate that ICI 182,780 acts on the antioxidant defense, whereby after treatment with ICI 182,780 there is an increase in the concentration of the PHGPx1/gpx4 mRNA, indicating the presence of a damage shelter (Guerriero et al. 2012), and this alters estrogen receptor expression in mammals (Gao et al. 2016). Our present results indicate that anti-estrogen treatment has a detrimental effect on spermatogenesis too via esr2 brain expression as already detected in the mammalian species by Oliveira et al. (2001). This allowed us to verify that by interfering with the maturation process with an endocrine disruptor treatment such as ICI 182,780 for 21 days, Podarcis sicula gonads and brain obtained esr2 levels closer to winter stasis. Assessing changes in estrogen receptor expression in animals exposed and not exposed to endocrine disruptor compounds is critical for our understanding of the role of these receptors in endocrine disruptors in the natural environment (Rie et al. 2005; Guerriero 2011; Guerriero et al. 2014; Verderame et al. 2014). In this study, not only has a histological alteration been noted, but also the return of expression levels of esr2 to values not significantly different from winter stasis for both the gonads and the brain. Thus, the role of esr2 in the control of reproductive function suggests its use as a biomarker of anti-estrogen damage.

Conclusions

In the current study, we show that the esr2 protein sequence of the lizard Podarcis sicula is evolutionarily very conserved, with a high sequence identity with the human ortholog. Furthermore, we report the transcription activity of esr2 by qRT-PCR in brain and testis in the main reproductive phases, i.e. during the period of maximum activity of gonads, and in winter stasis. The observed pattern will help us to both detect the normal feedback within the established brain-gonad dynamics when there is interference by anti-estrogen compounds, and to identify variability within this dynamic.

These findings will allow the use of esr2 as a biomarker in the bioindicator Podarcis sicula for biomonitoring environmentally restored sites, and further allow the development of quick and efficient tests by gonadectomy in pollution biomonitoring programs. This methodology may also be used for making predictions of risk based on future mathematical models. Certainly, the high conservation of the sequence of esr2 and the general cytological organization close to that of humans strongly suggest the use of Podarcis sicula as an excellent model to monitor the sexual maturation state following exposure to various substances, thus opening new horizons for the conservation of species, including the human species.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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