Reproductive Toxicology 57 (2015) 29–42

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Accumulation of organotins in seafood leads to reproductive tract abnormalities in female rats

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ARTICLE INFO

Article history:
Received 20 December 2014
Received in revised form 21 April 2015
Accepted 7 May 2015
Available online 4 June 2015

Keywords:
Environmental contamination
Organotins accumulated in seafood
Endocrine disruption
Reproductive toxicity

ABSTRACT

Organotins (OTs) are environmental contaminants used as biocides in antifouling paints that have been shown to be endocrine disrupters. However, studies evaluating the effects of OTs accumulated in seafood (LNI) on reproductive health are particularly sparse. This study demonstrates that LNI leads to impairment in the reproductive tract of female rats, as the estrous cycle development, as well as for ovary and uterus morphology. Rats were treated with LNI, and their reproductive morphophysiology was assessed. Morphophysiological abnormalities, such as irregular estrous cycles, abnormal ovarian follicular development and ovarian collagen deposition, were observed in LNI rats. An increase in luminal epithelia and ERα expression was observed in the LNI uteri. Together, these data provide in vivo evidence that LNI are toxic for reproductive morphophysiology, which may be associated with risks to reproductive function.
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1. Introduction

Organotins (OTs), such as tributyltin (TBT), are considered to be endocrine-disrupting environmental pollutants for sea and non-sea animals [1–3]. These xenobiotics are a diverse group of widely distributed organometallic contaminants, used as biocides in wood treatments and pesticides [4–6]; however, the most important use of TBT is in antifouling paints [1,7,8]. Environmental concerns regarding OTs have considerably increased due to their uncontrolled use, and consequently, high levels of TBT are found in harbors, waterways, and shipping lanes [9]. From previous studies, we learned that the level of TBT contamination in marine sediment may be classified into four categories, such as (i) lightly contaminated site (TBT levels of <0.01–0.05 μg/g), which is remote from boat moorings area; (ii) medium contaminated site (TBT levels varying between 0.06 and 0.2 μg/g), which is closer to moorings area; and (iii) highly contaminated site (TBT levels varying between 0.3 and 1 μg/g), within high density mooring areas [1,10].

OTs are known to be markedly toxic to oysters and molluscs, impairing reproduction in oyster *Crassostrea gigas* and inducing imposex in gastropod mollusks [1,11,12]. The mechanism of how OTs (such as TBT) lead to imposex is unclear; however, an association with the inhibition of aromatase, which converts testosterone into estrogen, acyl CoA-steroid acyltransferase inhibition; reduction of androgen excretion; abnormal release of APGWamide as results of TBT neurotoxic effect; or activation of retinoid X receptor appears to be involved [13–16]. Imposex is an endocrine syndrome where female molluscs develop male genitalia after exposure to OTs, mainly TBT. As result of TBT anthropogenic input in the environment, TBT can be found in marine biota and sediment (their adsorption was found mainly in fine marine sediments), varying between 2 and 20 years after its contamination [6,17,18]. As a result of TBT use in marine environments, its residues have been found in many organisms of food chains, including molluscs, fish, seabirds and marine mammals [19–22]. Furthermore, TBT has been shown to have highly toxic effects on non-target marine organisms, such as mammals [23–25].

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http://dx.doi.org/10.1016/j.reprotox.2015.05.003
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Several investigations have shown that consumption of seafood containing OTs is the primary source of human exposure in Asia, Europe and America [26–28]. Inorganic tin (iSn) is poorly absorbed by the gastro-intestinal tract (GIT) and is associated with OTs metabolization into iSn by mammals [29]. It has been suggested that an important fraction of iSn may be present in the bodies of mammals, as a result of OTs contamination, which strengthens the importance of the total tin determination for evaluating the exposure of mammalian to OTs [27,30,31]. In Brazil, monitoring studies have recorded the development of imposex in various gastropods species, such as *Stramonita rustica*, *Thais rustica* and *Voluta ebraea* [32,33]. In addition, in Espírito Santo State (ES), studies have shown that imposex occurs in *Stramonita haemastoma*, *Thais deltoidea* and *Leucozonia nassa* [34,35]. Recently, a high concentration of OTs in marine sediments was associated with the development of imposex in the mollusc *L. nassa*, reported in a harbor complex in Vitória Bay, ES, Brazil [36].

From previous toxicologic studies, Penninks [37] suggested for TBT, the tolerable daily intake (TDI) level for humans of 0.25 μg/kg and this TBT level proposed was adopted by the World Health Organization [37–39]. In addition, the U.S. Environmental Protection Agency proposed a TDI level of TBT slightly higher such as 0.3 μg/kg [40]. Interestingly, seafood samples collected from markets in Asian, European and North American cities presented TBT levels averaging 185 ng $(g\,dw)^{-1}$ [18]. The consumption of seafood containing OTs in Europe may lead to an OTs intake that exceeds the TDI of 0.27 μg/kg [41]. Similarly, TBT doses were estimated to be 2.6 μg TBT/day/person in Korean seafood [42,43]. Studies conducted in Brazil and Japan suggest the possibility of human health risks derived from the intake of OTs accumulated in seafood, especially in riparian populations [44,45]. The toxicology of OTs to humans is not fully understood [40,46]. Health risk assessments have largely been based on immunological studies in animal models or intake estimates of OTs in seafood sources [18,25,45,47,48]. In addition to those risks, mammalian studies have shown that OTs impair reproductive parameters, such as sexual hormone metabolism, estrogen receptor (ER) expression and its affinity by binder, gonadal development/function, uterine morphophysiology, pregnancy rate and others [23,24,49–53]. Despite these discoveries using the rodent models and health risk assessments, few studies have directly explored the effect of OTs accumulated in seafood to mammalian reproductive function. Therefore, in this study, female rat models were used to determine the effect of OTs present in seafood on the morphophysiologic impairment of reproductive organs.

2. Materials and methods

2.1. Experimental animals and treatments

In this study, the seafood used was mollusk *L. nassa* that were collected during 6 years (2007–2012, $n = 143$) inside Vitória Bay, ES, as the Frade Island, southeastern Brazil (77°54′S, 36°63′ W). This area was previously reported as a locality where imposex development was observed in *L. nassa* and it was exhibited high levels of imposex associated with OTs pollution in marine sediments [35,36]. Other *L. nassa* animals ($n = 100$) were collected in site free of OTs pollution, with grainy sediments (they were not able to accumulate OTs) and without shipping activity (control area, 19°49′S, 40°16′ W). The gastropod soft tissues with (imposex percentage ranging between 91 and 100%) and without (imposex percentage was 0%) imposex development were used to prepare a homogenate for the treatment of the female rats in different groups. Adult female Wistar rats (±230 g, 12 weeks old) were kept in cages with controlled temperature and humidity, 12 h light/dark cycles, and free access to water and food. The animals were weighed and randomly assigned to 3 treatment groups, and they were treated orally with either 0.5 mL distilled water (CON, $n = 10$), 600 mg day^{-1} homogenate of seafood with development of imposex and containing OTs (LNI, $n = 10$), or 600 mg day^{-1} homogenate of seafood without development of imposex and accumulation of OTs (LNN, $n = 10$). The oral route of exposure was chosen based on previous studies [3,24,53–56]. Using OTs accumulated in seafood enabled us to compare our results with previous work on OTs and allowed us to compare the effects of OTs present in seafood, which has strongly toxic properties as one of its potential mechanisms [44,45]. OTs accumulated in seafood are not a pure OTs; therefore, the use of seafood without OTs also enabled us to differentiate toxic from non-toxic effects. In addition, we did not find a similar study using seafood containing OTs and evaluating reproductive parameters. For this reason, we needed to compare the adverse effect found in our model with other toxicological studies that used pure OTs, such as TBT or triphenyltin (TPT) [3,24,57,58]. Furthermore, we evaluated the level of tin present in the seafood used in our model by an inductively coupled plasma mass spectrometer (ICP-MS). Also, in order to confirm the status of OTs contamination in marine sediments that were collected the seafood used (the same geographic area, 77°54′S, 36°63′ W), the levels of total butyltin compounds ($\sum$OTs), TBT, di-(DBT) and monobutyltin (MBT) were found as 970.0, 398 ± 9.0, 276 ± 8.6 and 296 ± 2.0 ng Sn g^{-1}, ($n = 3$, OTs were determined for us by gas chromatography with pulsed flame photometric detection, Varian GC 3800, Walnut Creek, CA, USA) [35,59], respectively, suggesting the maintenance of OTs contamination in this environmental site. In addition, we estimated that each rat was exposed to the dose of the seafood intake rate of Brazilian riparian communities (i.e., 200 g/day/70 kg) based on previous literature of human health risk assessments [44,45]. Therefore, our dose is estimated to be within the range of human exposure (in Brazil) to seafood containing OTs. The criterion for utilization of a 15 day treatment was based on a previously reported ovarian toxicity protocol [53,54]. The experimental procedures were in accordance with the Biomedical Research Guidelines for the Care and Use of Laboratory Animals [60], and the Ethics Committee for Animal Experiments of the Federal University of Espírito Santo (CEUA No. 047/10).

2.2. Tin assessment

To evaluate the tin concentrations in seafood, mollusks samples were collected and tin levels were assessment using an ICP-MS (NexIon 300-D, Perkin Elmer, Germany) [61]. Brief, seafood samples were digested with 30% of H_2O_2 (m/m) and ultra-pure HNO_3 (Elga - Purelab, Marlow, UK) using a microwave oven equipped with PTFE vessels (Multiwave 3000 microwave, Anton Paar, Graz, Austria). The analyses were carried out with an ICP-MS and the argon gas (99.999% Air Liquid, RJ, Brazil) was used in determinations of ICP-MS for plasma generation, nebulization and auxiliary gas. The sample introduction system was composed by cyclonic spray chamber and a Meinhard® nebulizer. The method's quality control section requirements were followed closely to demonstrate accurate quantification of tin in the seafood samples used in this study. The mass-to-charge ratio (m/z) was monitored with isotope $^{118}Sn^{+}$. The ICP-MS operating conditions were performed with generator frequency (40.0 MHz); plasma gas flow rate (16.0 l min^{-1}), auxiliary gas flow rate (1.20 l min^{-1}), nebulizer gas flow rate (1.02 l min^{-1}) and RF power (1.55 kW). The accuracy of the procedure was checked using reference sample (Seronorm Urine, Sero AS, Norway). The level of detection (LOD) for tin determined by ICP-MS was 4.0 ng g^{-1}. The levels of LOD were determined for each analysis performed.

Serum tin concentrations were also performed, blood samples were collected and tin levels were measured using a Model ZEEnit 700 atomic absorption spectrometer (Analytik Jena, Jena, Germany) equipped with a transversely heated graphite tube atomizer, and a Zeeman effect background correction system [31]. Briefly, the samples were weighed directly on the graphite platforms using an analytical microbalance and were introduced into the graphite tubes using a Model SSA 600 for automatic solid direct sampling. A tin hollow cathode lamp was used as a light source (Analytik Jena, Jena, Germany) and the measurements were performed at an integrated absorbance of 224.6 nm. In addition, we used Pd (10,000 $\mu g\,mL^{-1}$ Merck) and $MgNO_3$ (1000 $\mu g\,mL^{-1}$ SCP Science) (10 μg Pd + 6 μg $MgNO_3$) as a modifier that added to each measurement of a sample or standard, diluted in 0.2% (v/v) ultra-pure HNO_3 or water (Elga - Purelab, Marlow, UK). The results obtained are expressed in $ng\,g^{-1}$.

2.3. Estrous cycle assessment

The vaginal smears were collected daily at 10:00 a.m. for 15 days in 2- to 3-month-old rats. Smears were examined as stained preparations with hematoxylin and eosin (H & E), and observed with a light microscope to determine the stage of the estrous cycle [53]. The stage of the estrous cycle was classified as proestrus, estrus, or metestrus/diestrus based on observed ratios of cornified epithelial, nucleated epithelial and polymorphonuclear leukocytes as described in [62]. The frequencies of the estrous cycles and the days spent in the different phases were compared among the 3 groups (CON, LNN, and LNI rats). Others frequencies of the estrous cycles and the days spent in different phases evaluated were reported and included in the supplemental data 1.

2.4. Hormonal assays

To measure basal levels of serum LH, FSH, estrogen and progesterone, blood samples were collected from female rats after euthanasia, between 9:00 and 10:00 a.m. to avoid cycle-dependent LH surges that occur in the evening of proestrus. LH and FSH were measured using an Elisa assay (#122043 DA-LH152 and #120037 DA-FSH151 EIA, Diagnostic Systems Laboratories, Saronno, Italy). Estrogen and progesterone were measured using a radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA), as described by Podratz et al. [53] and Rodrigues et al. [55].

2.5. Morphological analysis

The animals were anaesthetized with ketamine (30 mg/kg, im) and xylazine (3 mg/kg, im) to collect various organs, and wet and dry weights were obtained. In addition, the uterine and ovary somatic index (organ weight/body weight) was calculated to determine the extent of organ hypertrophy (in diestrus) [53]. Ovaries and uteri were fixed in 10% buffered formalin phosphate solution (pH 7.4) and stored at 4 °C. Paraffin-embedded organs were sectioned at 5 μm thickness. Ovarian and uterine sections were stained with H & E, examined with high-quality images (2048 × 1536 pixels) in an Olympus microscope (AX70; Olympus, Center Valley, PA), photographed with an AxioCamICc1 camera and exported to AxioVision Software (AxioVision Rel. 4.8 software). For the ovary sections, ovarian follicles and corpora lutea (CL) were counted and expressed as units per area (mm^2), as described by Podratz et al. [53]. The ovarian follicles were classified according to the method of Myers et al. [63]. Briefly, the follicles were classified as primordial if they contained an oocyte surrounded by a partial or complete layer of squamous granulosa cells (GCs). Primary follicles showed a single layer of cuboidal GCs. Follicles were classified as preantral when they contained 2–4 layers of GCs with no antral space. Antral follicles contained three or more layers of GCs and a clearly defined antral space. The characteristics of atretic follicles included pyknotic GCs, disorganized GCs, degenerating oocytes and detachment from the basement membrane. In addition, the thickness of the uterine luminal epithelium (LE), endometrium (ENDO) and myometrium (MYO) were measured, as described by Mendoza-Rodríguez et al. [64]. The ovaries and uteri were analyzed by taking 5 regions of each of the 3 sections, resulting in a total of 15 measurements per animal. Two independent analyzers performed these observations.

2.6. Collagen density surface

Ovary sections stained with Picro-sirius red stain were used to obtain 15 photomicrographs from ovary tissue with a 400× magnification. The areas were randomly selected, although fields containing medium-sized blood vessels were carefully avoided [54]. The results were expressed as a percentage of the marked area.

2.7. Protein extraction and western blotting analysis

The ovaries and uteri (diestrus) were removed, homogenized in lysis buffer and equal quantities of protein were loaded into each lane (100 μg) of a 10% SDS-polyacrylamide gel, as described by Gracelli et al. [65]. Proteins were transferred to nitrocellulose membranes in Tris-glycine buffer (Bio-Rad Laboratories, Hercules, CA). The membranes were incubated overnight with 5% blotting-grade blocker non-fat dry milk in Tris-buffered saline plus 0.1% Tween 20 solution and specific antibodies (Bio-Rad Laboratories). The primary antibodies used were anti-ERα, (sc542; 1:500, SCBT, INC), anti-ERβ (sc8974, 1:500, SCBT, INC) and anti-β-actin (sc130656; 1:1000, SCBT, INC). Goat anti-rabbit IgG-alkaline phosphatase conjugate (A3687, Sigma–Aldrich, St. Louis, USA) was used as a secondary antibody for all blotting assays. The blots from ERα and ERβ and their respective β-actin proteins were visualized with a color development reaction using nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) (sc24981, SCBT, INC). The ERα, ERβ and β-actin bands were analyzed by densitometry using Image J software. The relative expression was normalized by dividing the ERα and ERβ values by the corresponding internal control values (β-actin).

2.8. Statistical analysis

Data are reported as means ± SEMs. The normality of the data (Kolmogorov–Smirnov test with Lilliefors correction) was tested. The assessment of OTs in marine sediments, seafood tin levels and serum tin levels are reported as the mean ± SD. Comparisons between groups were performed using a one-way analysis of variance (ANOVA) with a Tukey's post hoc test. Statistical significance was achieved when $p \leq 0.05$.

3. Results

3.1. Seafood used in LNI treatment has high tin levels

Seafood samples were collected in an area with and without OTs pollution and the seafood tin levels were determined using an ICP-MS. The distilled water used in CON treatment had tin levels similar or lower when compared to the LOD levels (LOD for tin determined using an ICP-MS was 4 $ng\,g^{-1}$, Fig. 1A). The seafood used to prepare LNN treatment had tin levels of 108.7 ± 0.2 $ng\,g^{-1}$, n = 3, Fig. 1A). In contrast, the seafood used to prepare LNI treatment presented higher tin levels when compared to the seafood used in

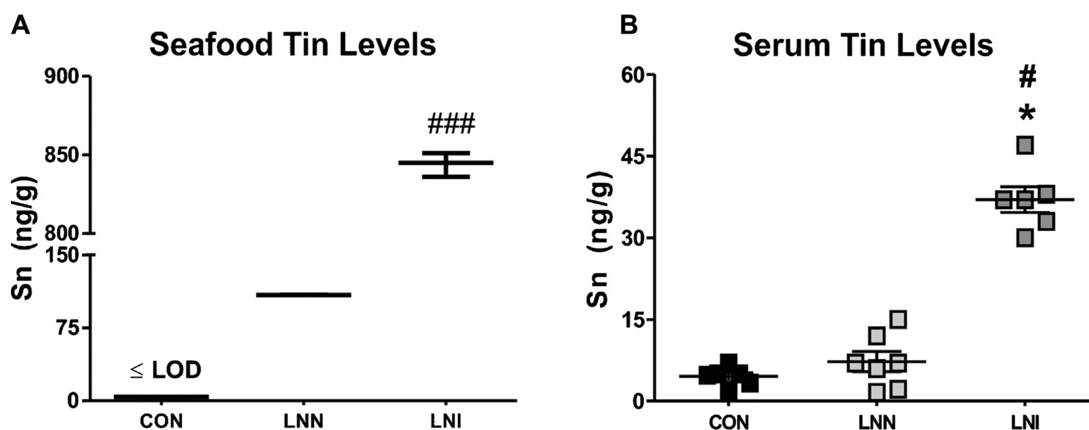

Fig. 1. Tin levels assessment in seafood and evaluation of serum tin levels of CON, LNN and LNI rats. (A) Seafood used in LNI treatment had higher tin levels ($n = 3$). (B) The LNI rats had raised serum tin levels ($n = 6$). Values are expressed as the mean ± SD $^*p \leq 0.05$ vs CON. $\#p \leq 0.05$, $\#\#\#p \leq 0.001$ vs LNN (one-way ANOVA followed by Tukey's test).

LNN treatment ($844.3 \pm 7.0\,\text{ng g}^{-1}$, $n = 3$, $p \leq 0.0001$, Fig. 1A). These data suggest the presence of high levels of OTs accumulated in the seafood used in LNI treatment.

3.2. LNI female rats have high serum tin levels

Serum samples were obtained from the CON, LNN and LNI rats and serum tin levels were determined using an atomic absorption spectrometer. The CON and LNN rats had tin levels of 4.0 ± 1.0 and $7.0 \pm 2.0\,\text{ng g}^{-1}$, respectively ($n = 6$, $p \geq 0.05$, Fig. 1B). In contrast, the LNI rats presented higher tin levels compared with the CON and LNN rats ($37.0 \pm 2.0\,\text{ng g}^{-1}$, $n = 6$, $p \leq 0.05$, Fig. 1B). This could be attributable to presence of high levels of OTs accumulated in the LNI seafood treatment.

3.3. LNI female rats have abnormal estrous cycles

Estrous cyclicity was evaluated in the CON, LNN and LNI female rats. Vaginal smears were collected daily for 15 days, and examined under a microscope to determine the stage of the estrous cycle (Fig. 2A). The CON and LNN rats had an average cycle length of 4.2 ± 0.3 and 4.3 ± 0.2 days, respectively ($n = 8$–10, Fig. 2B). In contrast, the LNI rats showed an absence of normal estrous cycling, varying between 5 and 8 days ($n = 8$–10, $p \leq 0.01$, Fig. 2B). The LNI rats were found in metestrus/diestrus 65% of the time, which was a significantly different pattern than observed in the control and LNN animals (Fig. 2B). Therefore, the LNI rats demonstrated an extensively abnormal pattern of estrous cyclicity.

3.4. Serum gonadotropin and sex hormones levels and somatic evaluation of the LNI rats

The pituitary weights of the LNI female rats was not different compared with the CON and LNN rats (Fig. 3A). Morning serum samples were collected from the CON, LNN and LNI rats and were used to determine FSH, LH, estrogen and progesterone hormone levels. Basal serum FSH and LH values in the LNI females were similar to those of the CON and LNN rats (Fig. 3B and C). In addition, an approximately 38% increase in the uterine somatic index was observed in the LNI rats (CON: 0.094 ± 0.006; LNN: 0.090 ± 0.006; LNI: 0.130 ± 0.008 mg/g b.w; n= $n = 7$–9, $p \leq 0.05$, Fig. 3D). However, the ovarian somatic index of the LNI, LNN and CON rats was not significantly changed (Fig. 3E). Further, the serum estrogen levels in the LNI females were significantly higher compared with the CON and LNN rats (LNI: 33.40 ± 1.79; CON: 9.91 ± 1.53; LNN: 7.8 ± 0.69 pg/mL, $p \leq 0.01$, $n = 7$–9, Fig. 3F). The serum progesterone values in the LNI females were also significantly higher compared with the CON and LNN rats (LNI: 6.94 ± 0.69; CON: 4.91 ± 0.30; LNN: 4.12 ± 0.42 ng/mL, $p \leq 0.01$, $n = 7$–9) (Fig. 3G). However, no significant changes were observed in the body, organ or tissue weights of the LNI rats compared with the CON and LNN rats (Table 1).

3.5. Reproductive tract abnormalities of LNI rats

The LNI ovaries exhibited morphological abnormalities with an impairment of ovarian follicular development (Figs. 4 and 5). The ovaries of the CON and LNN female rats displayed regular follicular development and normal corpora lutea (CL) (Figs. 4 and 5).

Table 1
Body, organ and tissue weight assessment. Values are expressed as the mean ± SEM.

Body/organ/tissue weights	CONT	LNN	LNI
Initial body weight (g)	201.62 ± 3.48	203.75 ± 9.82	209.87 ± 9.46
Final body weight (g)	219.25 ± 2.65	223.62 ± 9.18	235.87 ± 9.91
Heart (g)	0.64 ± 0.01	0.59 ± 0.03	0.67 ± 0.01
Heart dry weight (g)	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.01
Kidney (g)	1.41 ± 0.06	1.41 ± 0.06	1.55 ± 0.10
Kidney dry weight (g)	0.33 ± 0.01	0.32 ± 0.01	0.34 ± 0.02
Liver (g)	7.58 ± 0.52	7.37 ± 0.54	8.90 ± 0.80
Liver dry weight (g)	2.32 ± 0.16	2.27 ± 0.18	2.68 ± 0.23
Adrenal gland (g)	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Adrenal gland dry weight (g)	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Perirenal fat (g)	1.07 ± 0.27	0.75 ± 0.09	0.98 ± 0.13
Abdominal fat (g)	0.86 ± 0.22	1.08 ± 0.13	0.79 ± 0.08
Parametrial fat (g)	1.87 ± 0.16	2.43 ± 0.43	2.76 ± 0.38
Retroperitoneal fat (g)	1.29 ± 0.10	1.29 ± 0.19	1.72 ± 0.22

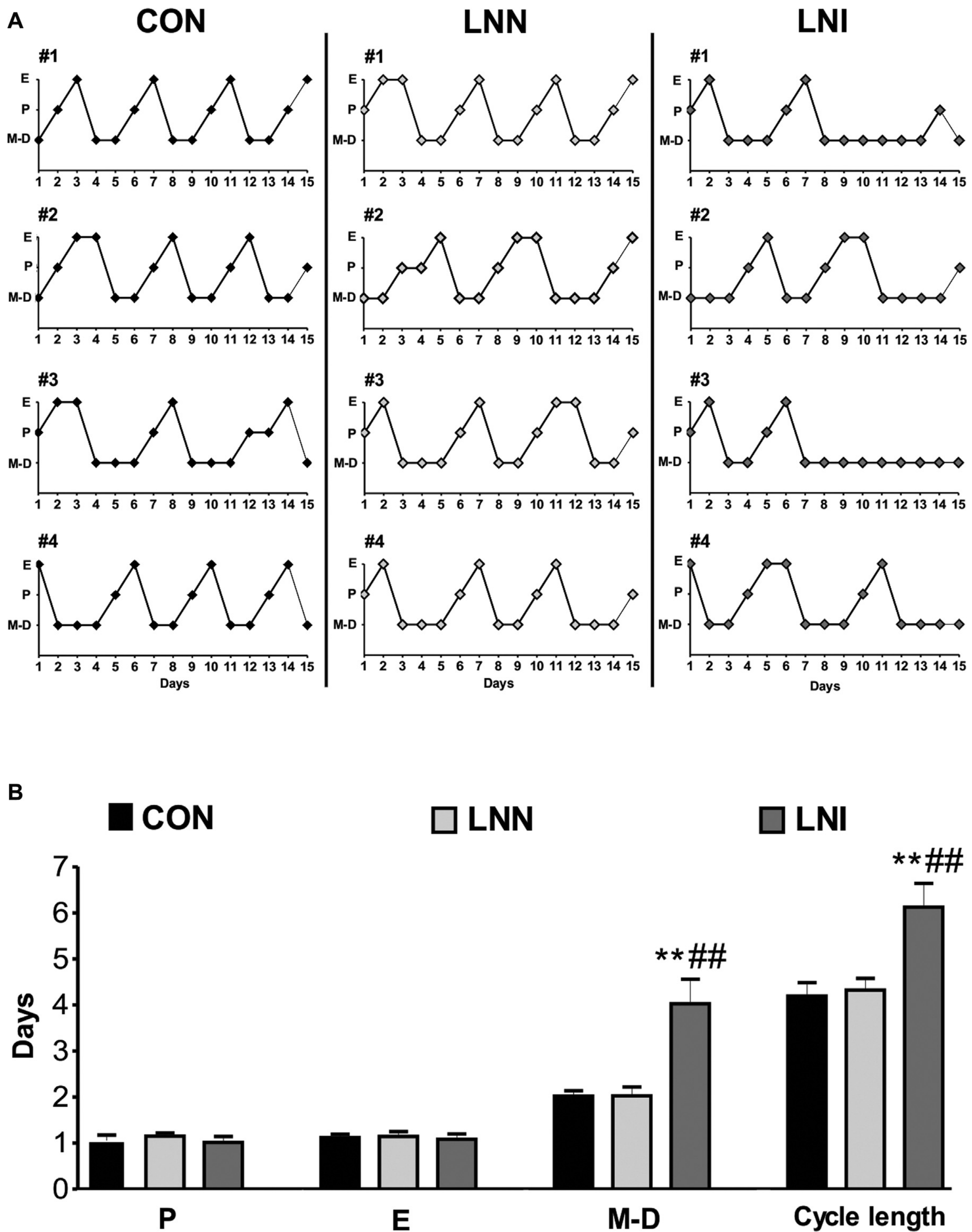

Fig. 2. Estrous cycle assessment determined by vaginal cytology observed for 15 days. (A) Graphic representation of the respective estrous cyclicity in the CON, LNN and LNI rats. The LNI rats had an abnormal estrous cycle. (B) Graphic representation of the number of days in each stage of the estrous cycle and cycle length. The LNI rats had raised estrous cycles. P, proestrus; E, estrus; M-D, metestrus–diestrus. Values are expressed as the mean ± SEM ($n = 8–10$). $^{**}p \leq 0.01$ vs CON. $^{\#\#}p \leq 0.01$ vs LNN (one-way ANOVA followed by Tukey's test).

However, the LNI rats showed the presence of cystic ovarian follicles, apoptotic cells in the CL and antral ovarian follicles with disruption in oogenesis (Fig. 4G–I). The LNI ovaries had an increased number of primary ovarian follicles compared with the CON and LNN ovaries (CON: 1.14 ± 0.11; LNN: 1.18 ± 0.08; LNI: 1.8 ± 0.13 no./mm^2, $n = 5$, $p \leq 0.05$, Fig. 5B), though the number of primordial ovarian follicles was not significantly changed (Fig. 5A). In addition, an approximately 144% increase in the number of cystic ovarian follicles was observed in the LNI ovaries (CON: 1.21 ± 0.11; LNN: 1.15 ± 0.06; LNI: 2.93 ± 0.22 no./mm^2, $n = 5$, $p \leq 0.01$). An increase in the number of atretic ovarian follicles in the LNI was observed compared with the CON and LNN ovaries (CON: 0.75 ± 0.10; LNN: 0.97 ± 0.18; LNI: 1.67 ± 0.30 no./mm^2, $n = 5$, $p \leq 0.05$, Fig. 5E). In addition, a raise in the quantity of CL in the LNI was observed

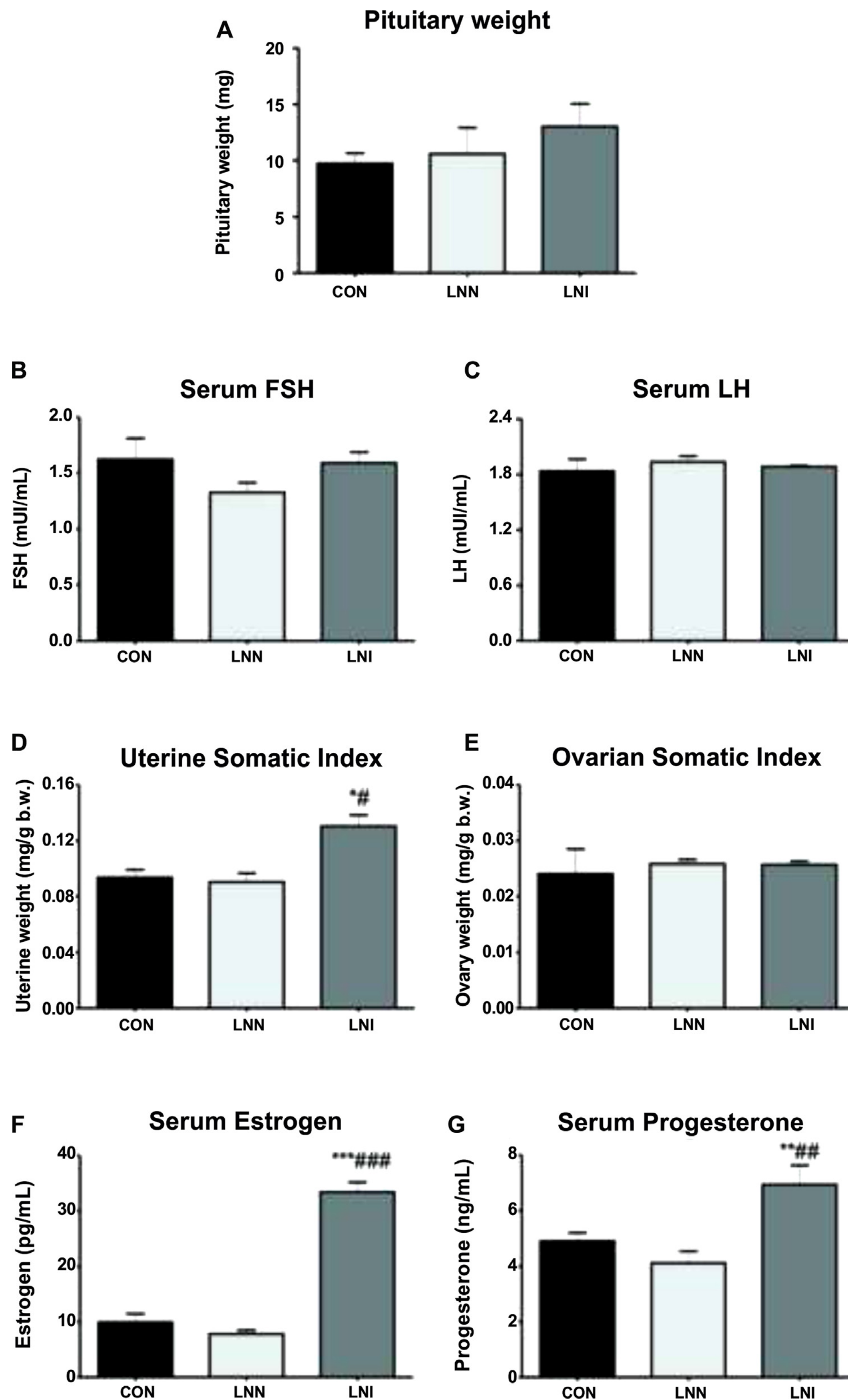

Fig. 3. Serum gonadotropin and sex hormone levels and somatic marker evaluation of the CON, LNN and LNI rats. (A) Pituitary weight assessment. (B) Serum FSH levels. (C) Serum LH levels. (D) Uterine somatic index. (E) Ovarian somatic index. (F) Serum estrogen levels. (G) Serum progesterone levels. Values are expressed as the mean ± SEM ($n = 7–9$). *$p \leq 0.05$, **$p \leq 0.01$,***$p \leq 0.001$ vs CON. #$p \leq 0.05$, ##$p \leq 0.01$, ###$p \leq 0.001$ vs LNN (one-way ANOVA followed by Tukey's test).

compared with the CON and LNN ovaries (CON: 0.81 ± 0.09; LNN: 0.96 ± 0.13; LNI: 1.27 ± 0.13 no./mm^2, $n = 5$, $p \leq 0.05$, Fig. 5F). However, no changes in the numbers of preantral and antral ovarian follicles were observed between the groups analyzed (Fig. 5C and D).

The development of fibrotic processes was assessed in the CON, LNN and LNI ovaries. The collagen deposition in the cortical and medullar space was significantly higher in the LNI ovaries, especially at the region around the ovarian follicles, as shown by the

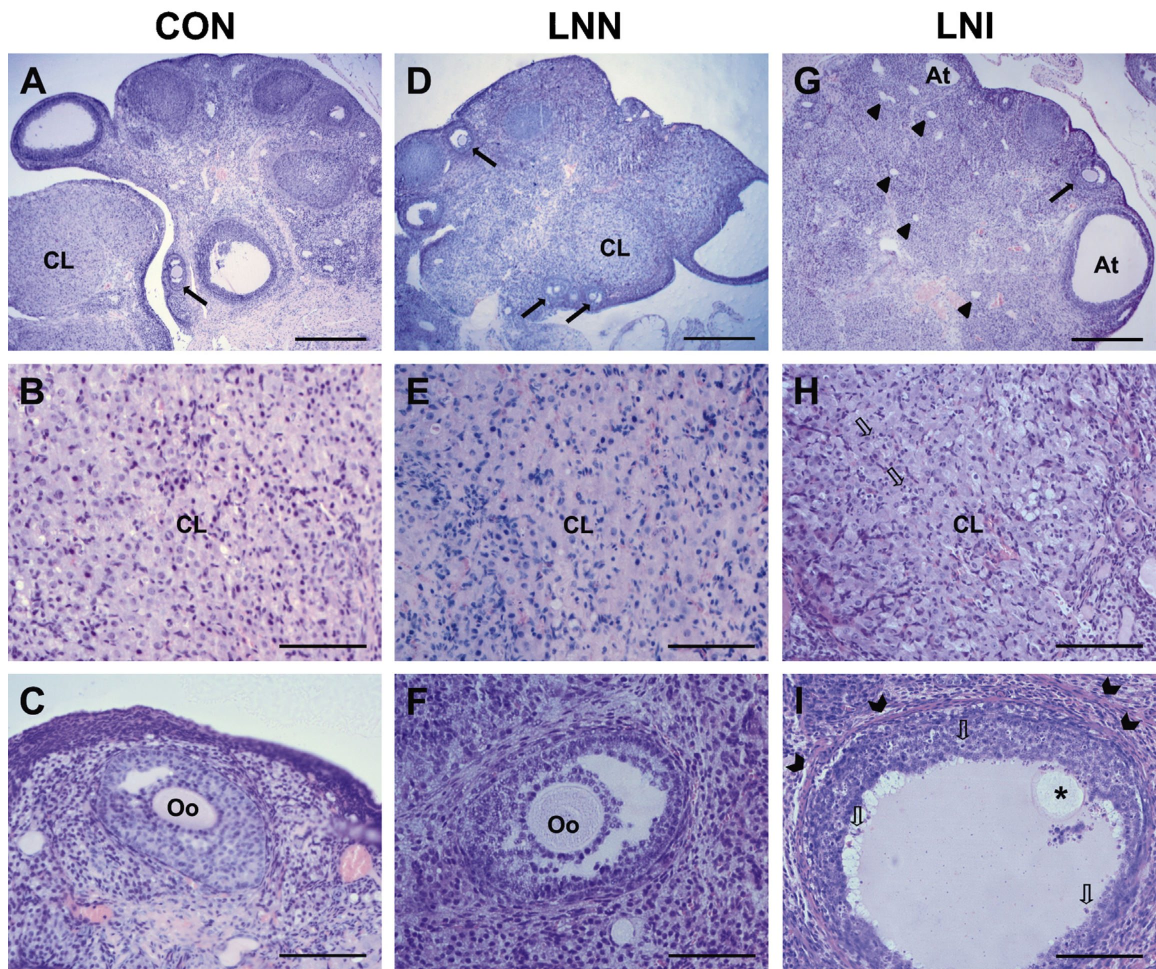

Fig. 4. H & E ovary stained sections from the CON, LNN, and LNI rats. Representative sections from a (A) CON and (D) LNN ovary showing follicles at all stages of development, including primordial, primary, preantral, antral (arrow) follicles as well as corpora lutea (CL). (B) CON and (E) LNN CL presenting normal aspects. (C) CON and (F) LNN antral follicles showing normal structure and intact oocyte (Oo). (G) LNI ovary exhibiting antral and atretic follicles (At); notice the presence of the cystic follicles (arrowhead). (H) LNI CL exhibiting apoptotic cells (arrow unfilled). (I) Atretic antral follicle with highly vacuolated oocytes (asterisk), and dead cells (arrow unfilled) in the granulosa cells and in the antral space. In addition, collagen fibers were observed around the ovarian follicles and stroma (arrowhead). Image A, D and G (bar: 400 μm); B, C, E, F, H and I (bar: 100 μm) ($n = 5$).

Picro Sirius Red staining (CON: 2.59 ± 0.30; LNN: 3.30 ± 0.41; LNI: $10.80 \pm 0.77\%$, $n = 5$, $p \leq 0.05$, Fig. 6).

The uteri also exhibited morphological differences in the CON, LNN and LNI rats (Fig. 7). The thickness of the uterine luminal epithelia (long cylindrical cells) was significantly more in the LNI compared with the CON and LNN uteri (CON: 14.44 ± 0.80; LNN: 16.71 ± 0.65; LNI: 19.75 ± 1.05 μm; $p \leq 0.05$, Fig. 7D), suggesting epithelia with pseudostratification. However, the thickness of the endometrium and myometrium were not significantly different between the CON, LNN and LNI groups (Fig. 7E and F).

3.6. Ovarian and uterine ERα and ERβ protein expression in LNI rats

ERα and ERβ protein expression was evaluated in the CON, LNN and LNI ovaries and uteri using an immunoblotting assay. There were no differences in the ERα and ERβ protein expression in the ovaries of the CON, LNN and LNI (Fig. 8A and C). However, uterine ERα protein expression was 30% higher in the LNI rats compared with the CON and LNN rats (CON: 1.00 ± 0.02; LNN: 1.03 ± 0.07; LNI: 1.34 ± 0.15, $n = 5$, $p \leq 0.05$, Fig. 8B). In addition, there was no difference in the ERα protein expression of the CON or LNN uteri. Further, the uterine ERβ protein expression was similar in the CON, LNN and LNI (Fig. 8D).

4. Discussion

Endocrine disrupting chemicals (EDC) have become a prototypical model for disease development, with exposures during critical developmental periods associated with dysfunctions related to metabolic status, reproductive, thyroid and many other systems and/or organs. However, little of this research has extended the seafood contamination model to the toxicology of OTs, and an even smaller subset has focused on reproductive toxicology. Several investigations have reported a possible health risk in the consumption of seafood containing OTs [44,45,66]. Available information on OTs deposition in humans or mammals, based on a few studies in Japanese, Polish or Danish populations, is limited [18,48,67].

This lack of research of the effect of OTs led us to investigate additional deleterious effects of these xenobiotics accumulated in seafood in our model reproductive tract assessment. Moreover, this study is the first to reveal that the toxic effects of accumulated OTs in seafood appear to be responsible for the disruption of ovarian and uterine morphophysiology leading to abnormal estrous cyclicity, ovarian sexual hormone imbalance and an increase in ovarian collagen deposition.

The current study sought to determine whether exposure of female rats to OTs accumulated in seafood would impair the reproductive tract through the toxicity of OTs to reproductive function.

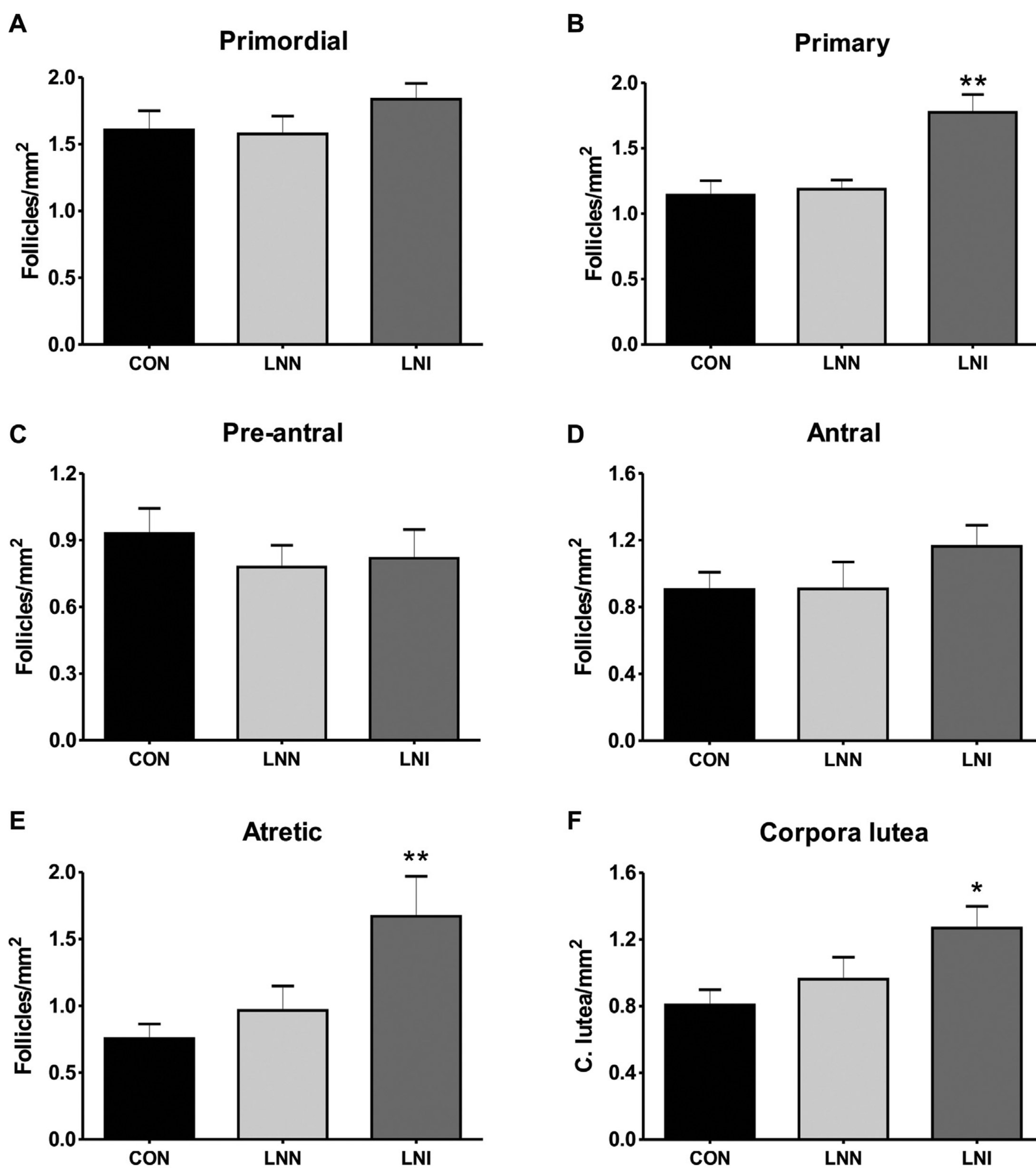

Fig. 5. Ovarian follicles and CL counts per unit area in the CON, LNN and LNI ovaries (number/mm^2). (A) Primordial, (B) primary, (C) pre-antral, (D) antral, (E) atretic follicles and (F) CL. Values are expressed as the mean ± SEM ($n = 5$). $^*p \leq 0.05$ and $^{**}p \leq 0.01$ vs CON (one-way ANOVA followed by Tukey's test).

Thus, we assayed ERs expression and morphophysiology in the ovary and uterus. The foundation for this work is the body of epidemiological evidence suggesting that there are correlations between exposures to some classes of EDCs (notably OTs such as TBT) and reproductive toxicology in mammals [3,23,24,53,68]. However, some of these results have been contradictory, possibly due to differences in the timing of exposure, dose, and type of EDC, as well as in differences in populations or animal models [3,24,53,68,69].

A seafood tin assessment was performed to determine the status of OTs accumulation in the seafood used in LNN and LNI treatment. Previous studies reported that seafoods, such as fish, shellfish and mollusks found in area contaminated with OTs were able to accumulate OTs in their tissues [22,70,71]. Our results agree with the previous findings, we observed higher tin levels in the seafood used in LNI treatment. Interestingly, Takahashi et al. [72] reported that the levels of total butyltin compounds and total tin presented the same behavior concerning body distribution, differentiation between coastal and offshore species, as well as age trends in accumulation of marine mammals collected from Japanese coastal area. Therefore, it can be suggested that a tin significant accumulation present in the seafood's body may is a result of OTs exposure from the environmental source. In addition, a serum tin assessment was performed to determine the status of OTs contamination in the LNI rats. It is known that the liver is involved in the biotransformation of exogenous xenobiotics into water-soluble products, which are excreted into the extracellular medium [73]. For instance, TBT accumulates in the mice liver and it appears to be metabolized by cytochrome P450 to DBT> MBT> and iSn [74]. Here, we observed that treatment with OTs accumulated in seafood increased serum tin levels in the LNI rats. This was similar to the findings of Dornelles et al. [31], considering that intake of iSn is poorly absorbed by mammalian GIT and they are able to metabolize OTs into iSn [29]. Thus, it can be suggested that an important fraction of iSn present in the mammal's body is a result of exposure to OTs or their intake in seafood as reported in this study for LNI rats.

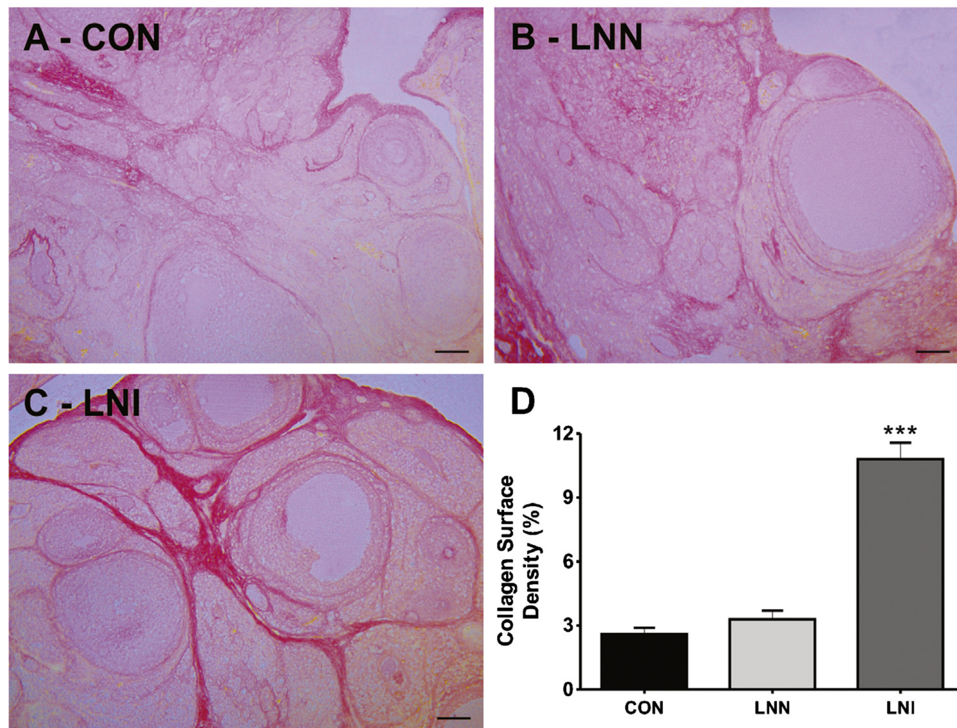

Fig. 6. Density of collagen deposition in ovaries of the CON, LNN and LNI rats using Picro-sirius red staining. Representative images of (A) CON, (B) LNN and (C) LNI ovaries. OTs accumulated in seafood increased collagen deposition in the LNI ovaries. (D) Graphic representation of collagen surface density in the CON, LNN and LNI ovaries. Images A, B and C (bar: 100 μm). Values are expressed as the mean ± SEM ($n = 5$). ***$p \leq 0.001$ vs CON (one-way ANOVA followed by Tukey's test).

Previous studies have reported that OTs play a direct role in the development of toxicology and improper functioning of the reproductive organs in both genders [23,24,49–51,53] (Table 2). Several studies have shown that OTs exposure in mammalian models or fetal exposure of OTs results in various degrees of endocrine disruption, as well as the dysfunctional development of sexual organs [24,49–51,53]. Therefore, a potential health risk may exist for heavy consumers of OTs accumulated in fish or other seafood, as reported to the general populations of Taiwan or in Brazilian riparian communities [44,46,75,76]. In vitro studies using human blood cells reported an immunotoxic effect to OTs doses similar to those present in the blood analysis of 38 volunteers from Michigan, USA (155 $\mu g\,L^{-1}$) [48,77,78]. In agreement with these previous toxicological findings, the LNI rats displayed distinct evidence for reproductive toxicology; an abnormal estrous cyclicity was observed in the LNI females, with most of them in diestrus phases, suggesting a disruption in normal ovary function. A common feature of exposure to OTs in rodents is ovary dysfunction of the female reproductive tract [24,50,51,53]. In addition, abnormal estrous cycles with a change in the length of the phases was observed in older rodents through changes in both ovarian and hypothalamic-pituitary hormones [79,80].

Table 2
Summary of reproductive changes induced by OTs and their accumulation in seafood.

Reproductive parameter	Animal/OTs/dose						
	Rat/TBT (0.25–20 mg/kg)	Mice/TBT (0.001–0.1 mg/kg)	Rat/TPT (6 mg/kg)	Rat/DBT (3.8–15.2 mg/kg)	Seafood (OTs)	Humans (seafood)	Rat/seafood (2900 mg/kg)
Source of exposure	Chemical (induced)	Chemical (induced)	Chemical (induced)	Chemical (induced)	Environmental (shipping traffic areas)	Intake (freely)	Environmental (induced)
Body weight	↓/↑	↔	↔	NR	NR	NR	↔
Vaginal opening	NR	↓	↔	NR	NA	NR	NR
Estrous cycling	NR	Impaired	NR	NR	NA	NR	Impaired
Pituitary weight	NR	NR	NR	NR	NA	NR	↔
LH levels	NR	NR	NR	NR	NA	NR	↔
FSH levels	NR	NR	NR	NR	NA	NR	↔
Estrogen levels	NR	↔	↑	NR	NA	NR	↑
Progesterone levels	NR	NR	↔	NR	NA	NR	↑
Ovary foliculogenesis	NR	NR	NR	NR	NA	NR	Impaired
Ovary morphology	Impaired	↔	NR	NR	NA	NR	Impaired
Uterine morphology	NR	NR	NR	NR	NA	NR	Impaired
Fertility	NR	NR	NR	Impaired	NA	NR	NR
Reference	Kishta et al. [51] Cooke et al. [109]	Si et al. [58]	Grote et al. [24]	Ema and Harazono [110]	Costa et al. [35] Santos et al. [66] Ho and Leung [111]	Kannan and Falandyzy [47] Kannan et al. [48] Lo et al. [112]	This study

OTs: organotins; TBT: tributyltin; TPT: triphenyltin; DBT: dibutyltin; ↑: increased; ↓: decreased; ↔: unchanged or similar to control; NR: not reported; NA: not applicable.

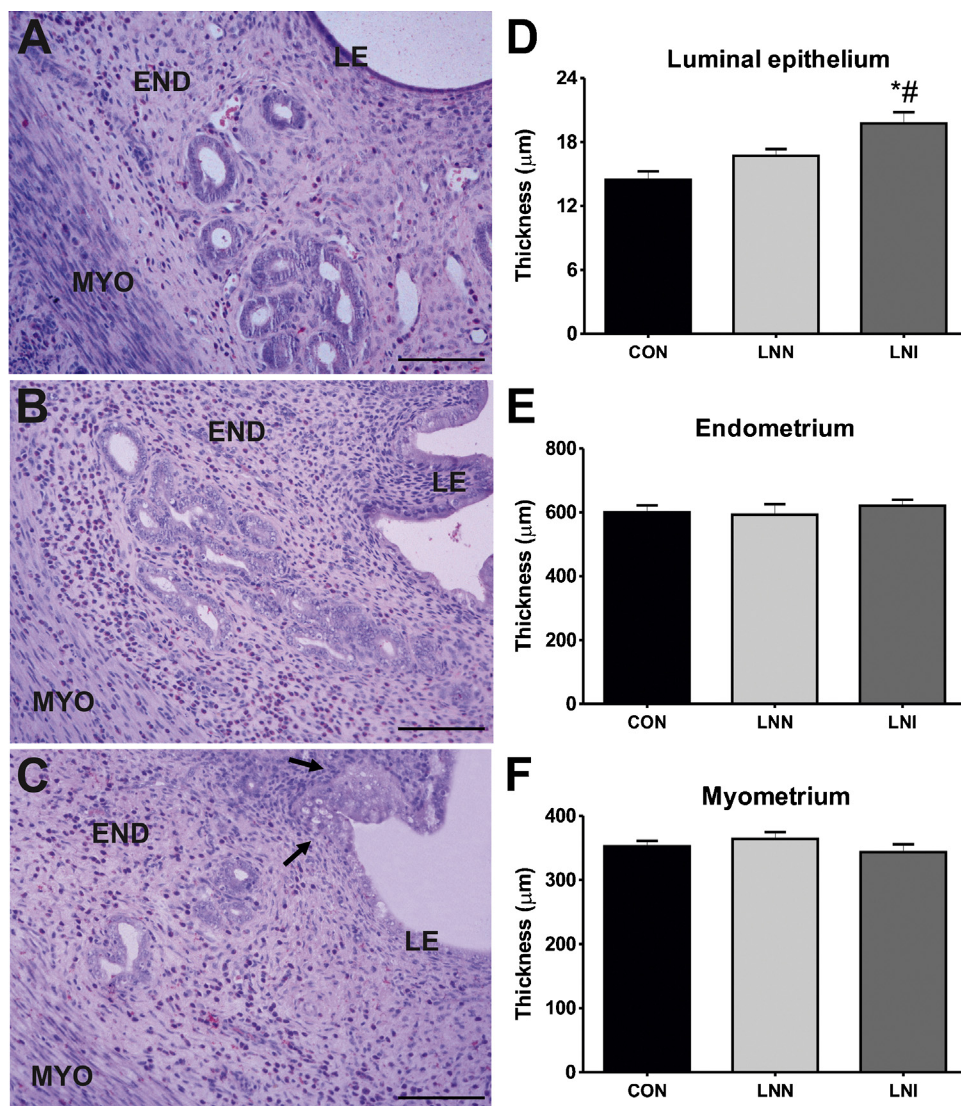

Fig. 7. H & E uterus stained sections from the CON, LNN and LNI rats. Representative sections from a (A) CON and (B) LNN uterus showing a typical thickened uterine luminal epithelium (LE), endometrium (END) and myometrium (MYO). (**C**) LNI uterus showed pseudostratified hyperplastic/hypertrophic (arrow). (D) Representative graph of the thickness of LE, with an increase in the LNI uterus. (E) Representative graph of the thickness of END. (F) Representative graph of the thickness of MYO. Images A, B and C (bar: 50 μm). Values are expressed as the mean ± SEM ($n = 5$). $^{*}p \leq 0.05$ vs CON. $\#p \leq 0.05$ vs LNN (one-way ANOVA followed by Tukey's test).

In addition, ovarian follicular development was impaired in the LNI females with the presence of cystic follicles, and increased primary follicles, atretic follicles and CL, indicating an imbalance of sex hormones in these rat models. The mechanisms responsible for ovarian follicular development and the paracrine signals that permit variable timing of ovarian follicle activation are completely unknown [81,82]. Numerous factors have been implicated in the regulation of the early follicle development, such as ovarian steroid levels, rodents strains or others [82–84]. Also, estrogen sensitivity difference was observed in the ovarian follicular development of diverse mice strains [84]. Kezele and Skinner [83] reported that the treatment of estrogen or progesterone increased the percentage of primordial ovarian follicles in postnatal 0-day-old rat ovaries in culture assay. However, the combination of estrogen and progesterone treatment displayed no changes the proportion of primordial ovarian follicles assessment. In another study, an increase in number of primary ovarian follicles without any change in the number of primordial ovarian follicles was observed in bovine ovarian cortex from fetuses at 91–140 days of gestation 5α-dihydrotestosterone-treated [82]. Furthermore, it is possible that estrogen may have indirect effects on primordial or primary ovarian follicles through the paracrine factors such as the growth differentiation factor 9 (GDF9), has been shown to increase primordial ovarian follicle development [85,86]. Thus, abnormal control of ovarian follicular development can lead to abnormal conditions such as premature ovarian failure or others ovarian dysfunctions, as observed for us. Moreover, in agreement with our findings, an increase in the number of atretic and cystic follicles was also observed in the ovaries of female pubertal rodents exposed to 6 mg of TPT and 100 μg/kg of TBT [57,58]. Kishta et al. [51] described a 44–46% reduction in ovarian germ cells in mice treated between 0–19 and 8–19 days of gestation at 20 and 10 mg/kg of TBT doses. Saitoh et al. [69] described cell death and apoptosis in human granulose-like tumor cells within 24 h of treatment at 1000 and 200 ng/mL doses of TBT. The genotoxicity process can result from a variety of factors including radiation, toxins, chemicals, xenobiotics, endocrine disruptors and reactive oxygen species [87–89]. DNA damage or mutagenic effects could be responsible for the improper functioning of the reproductive system or the appearance of tumors [53,69,90,91].

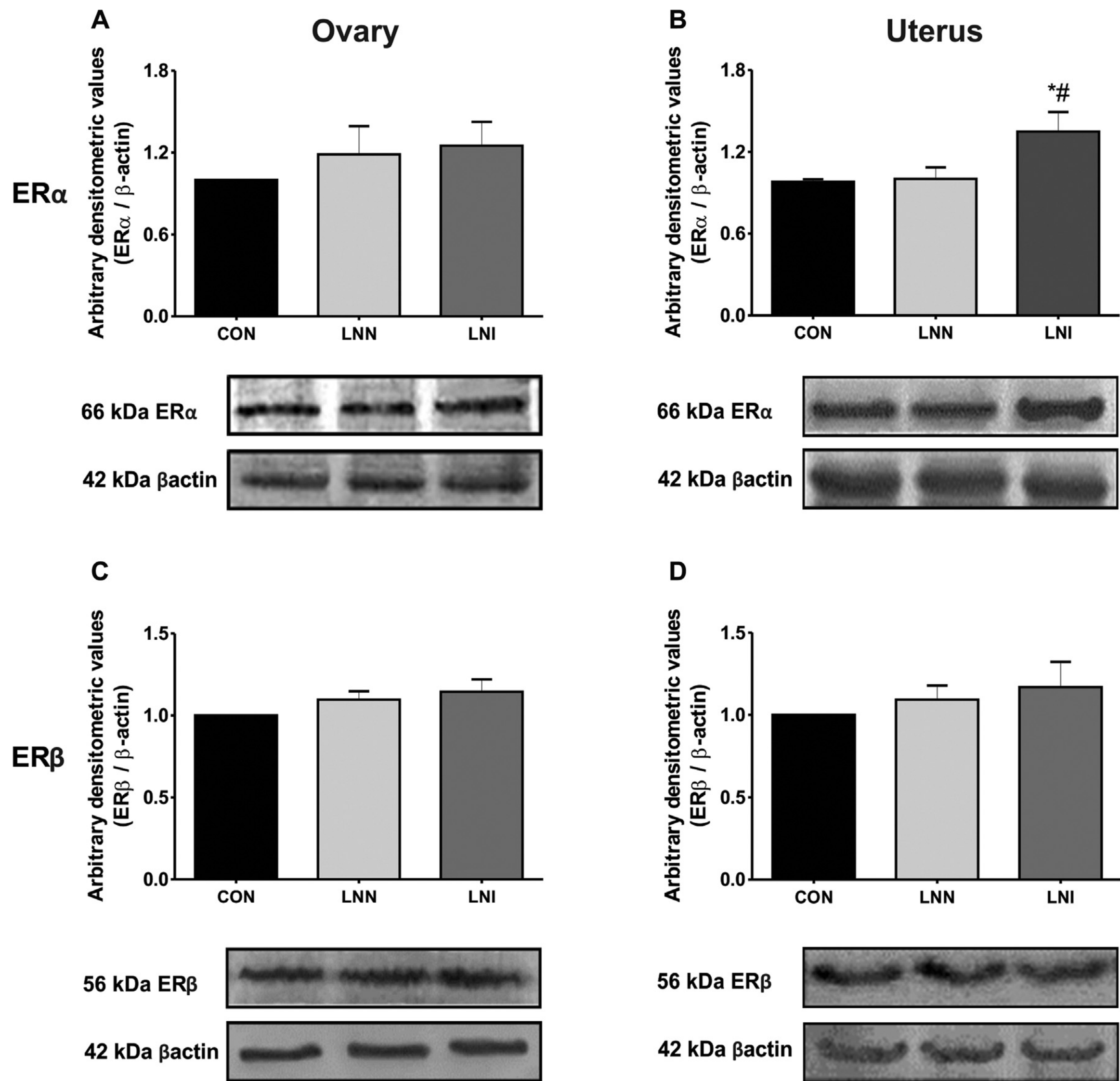

Fig. 8. Western blot analysis for ERα and ERβ proteins in the (A and C) ovaries, and (B and D) uteri of the CON, LNN and LNI rats. The results are expressed as the mean ± SEM ($n = 5$). $^{*}p \leq 0.05$ vs CON. $\#p \leq 0.05$ vs LNN (one-way ANOVA followed by Tukey's test).

One of the key characteristics of ovarian dysfunction and premature failure, which has been associated with an intense collagen deposition, is polycystic ovarian syndrome [92,93]. Here, the LNI rats had an increase in collagen deposition in their ovaries, demonstrating the improper functioning of ovary physiology, as well as in morphological architecture. Hatzirodos et al. [94] reported high levels of collagen and TNFα expression in atretic ovarian follicles of non-pregnant cows. Moreover, TBT is known to have a direct effect on the increase of protein TNFα and TNFR1 expression in rat ovarian cells after exposure at 10 mg/kg TBT [95]. Furthermore, OTs led to dysfunction during steroidogenesis and the abnormal ovarian follicular development could be attributable the binding of TNFα to TNFR1, activating apoptosis pathways by cleaved caspases [95–98].

Previous toxicological studies have shown that rats exposed to TBT display a drastically altered hormonal signature [3,24,53]. Nakanishi et al. [90] reported an increase of aromatase activity (AA) and estrogen production in human placental choriocarcinoma cells after TBT treatment for 48 h (0, 1, 10 and 100 nM). Our results agree with previous findings; the treatment with OTs in seafood increased serum estrogen levels in female rats. However, the effects of OTs in ovarian steroidogenesis are controversial. Grote et al. [24] found that TBT decreased ovarian AA and increased estrogen levels in PND 53 female rats, although the same effect was not observed in PND 33 female rats. These findings could be attributable to abnormal feedback mechanisms in the hypothalamic–pituitary–ovary axis caused by the treatment with OTs accumulated in seafood [3,24,25]. Therefore, the dramatic nature of this finding is not completely understood.

It is known that estrogen plays a role in the regulation of normal reproductive tract function mediated, principally, by nuclear ERs (ERα and ERβ) [99]. Principal estrogenic effects in the uterus are mediated by ERα, which is the predominant ER found in uterine tissue, regulating epithelial morphogenesis and uterine development [100–102]. In addition, uterine luminal epithelium (LE) height is a classic marker of high estrogen levels [103]. A previous study showed that a treatment of herbicide pendimethalin (600 mg/kg/day) led an increase in rat uterine weight and mRNA ERβ expression, although the uterine mRNA ERα and progesterone receptor expressions were not affected [104]. Additionally, exposure to bisphenol-A (BPA, 100 μg/day) did not alter ERα expression in the uterus and pituitary gland; however, it increased ERα expression in the hypothalamus of PND1-5 rats [105]. Here, the LNI rats had an increase in ERα protein expression and a thickening of LE in the uteri. Schönfelder et al. [101] reported an increase

of ERα immunostaining in rat offspring exposed to 50 mg/kg/day BPA. In addition, the uterine ERα mRNA expression was higher in rodents treated with estrogen varying between 50 μg/kg and 4 mg/kg [106,107]. Uterine hypertrophy/hyperplasia and pseudostratification of the LE have been observed with xenobiotic exposure to OTs, phytoestrogen genistein and BPA [51,64,108].

In conclusion, adult exposure to EDCs can disrupt reproductive parameters and result in the impairment of normal reproductive function. Our study adds insight into these processes by characterizing the morphophysiological changes in ovarian and uterine tissues, ER expression and imbalances in sex hormones following OTs exposure in seafood. This work aids the understanding of OTs. toxicity from seafood to the mammalian reproductive tract and points to the health risk of consuming OTs accumulated in seafood in the development of reproductive abnormalities in mammals.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

This research supported by Ciências Sem Fronteias (#18196-12-8), FAPES (#45446121/2009-002) and UFES (#PIVIC 2010-11).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2015.05.003

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