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Risk assessment and environmental consequences of the use of the *Allium*-derived compound propyl-propane thiosulfonate (PTSO) in agrifood applications

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ABSTRACT

The organosulfur compound propyl-propane thiosulfonate (PTSO), mainly found in *Allium cepa*, has a promising use in the agrifood industry. To confirm its safety for livestock, consumers, and environment, toxicological assessment is needed. In this regard, endocrine-disrupting chemicals (EDCs) are in the spotlight of research. Therefore, as part of the risk assessment of PTSO, in the present work, an *in vivo* study was performed in mice exposed to PTSO to investigate its potential reproductive toxicity considering fertility, genetic and endocrine endpoints. Five-weeks-old CD1 mice (80 males, 80 females) were exposed for 11 or 16 weeks (males or females, respectively) to different doses of PTSO (0, 14, 28 and 55 mg PTSO/kg b.w./day; 20 animals per group and sex) through the food pellets. No clinical observations or mortality and no changes in absolute organ weights and relative organ weights/body weight or brain ratios occurred during the study. The estrous cycle did not undergo any significant toxicologically relevant change. Most of the sex hormones displayed normal values. Some alterations in the expression of some genes related to reproduction is only observed in females, but they do not appear to have consequences in the development of sex organs. Docking results showed the impossibility of stable binding to estrogen and androgen receptors. Considering all the results obtained, the safe profile of PTSO can be confirmed for different agrifood applications at the conditions assayed.

1. Introduction

Recent research has demonstrated the diverse biological applications of natural bioactive compounds in the agrifood industry (Bravo and Lillehoj, 2013; Aguinaga-Casañas et al., 2022; Cabello-Gómez et al., 2022; Cascajosa-Lira et al., 2023a). Organosulfur compounds (OSC) are well-known molecules for their functional properties including, flavorings, antimicrobial, antioxidant, anti-inflammatory, among others (Sorlozano-Puerto et al., 2018; Putnik et al., 2019; Vezza et al., 2019; Farhat et al., 2021). In this sense, the number of patents and research on OSC as a technological additive has increased in the last decade. Hence, dialkyl thiosulfinate or thiosulfonate was used to decrease apicomplexa in animals (Bravo and Lillehoj, 2013). Similarly, propyl-propane thiosulfinate (PTS) and propyl-propane thiosulfonate (PTSO) have proved to be useful to prevent and reduce parasites in aquatic animals, contributing to the removal of residues generated by antiparasitics and antibiotics in the environment (Baños-Arjona et al., 2016).

Moreover, this OSC was proposed for different applications in the agrifood sector such as a sensory additive in animal nutrition or biopreservative in food packaging (Peinado et al., 2012, 2013; Llana-Ruiz-Cabello et al., 2015).

PTSO, a compound derived from onion (*Allium cepa*) is formed from the disproportionation reaction of PTS, which, in turn, is generated through the reaction of alliinase with propiin (Guillamón et al., 2021). Taking into account the environmental consequences of PTSO and its metabolites, its potential ecotoxicity was assessed *in silico,* and showed

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that the parent compound was detoxified (Cascajosa-Lira et al., 2023b). Moreover, despite several *in vivo* experimental studies were already performed to demonstrate the toxicity absence of PTSO: genotoxicity study (Mellado-Garcia et al., 2016) and subchronic 90-days study (Cascajosa-Lira et al., 2020) in Sprague-Dawley rats, further studies are necessary to have a complete overview of its toxicological profile.

Over the last four decades, there was an increase in the research of xenobiotics that can modify the endocrine system of humans and other vertebrates, and invertebrates (Cunha et al., 2022; Macedo et al., 2023). Endocrine-disrupting chemicals (EDCs) are exogenous compounds (or mixtures) that are able to disrupt the homeostasis of the endocrine system in healthy organisms or their progeny, by interfering with hormone actions (Zoeller et al., 2012; Gore et al., 2015; Demeneix and Slama, 2019; Gupta et al., 2022). EDCs can interfere with the production, release, transport, metabolism, binding, or elimination of natural hormones in the body, which can have negative effects on human and animal health and development (WHO/IPCS, 2002). In the beginning, research on EDCs was mainly focused on xenobiotics that act on the estrogen receptor and therefore modulate estrogen activity as agonists or antagonists. However, nowadays different types of substances have been reported to interfere with the endocrine system through several mechanisms (Metcalfe et al., 2022). The impact of EDCs on human health and the environment remains a global challenge and represents a concern in the EU (European Commission, 2022).

Exposure to EDCs has led to deleterious health effects, such as neurobehavioral changes, reproductive abnormalities, neoplastic lesions, and immunological malfunction (McKinlay et al., 2008; WHO, 2013; Encarnacao et al., 2019). Molecules with endocrine activity include pesticides, plasticizers, industrial chemicals, natural androgens and estrogens, pharmaceuticals and personal care products used for industrial applications, and illicit drugs (Metcalfe et al., 2022). At the present time, it is widely accepted that EDCs of anthropogenic origin are chemically diverse, ubiquitous in the environment, and that exposure of wildlife and humans to multiple chemicals is occurring by food intake (Metcalfe et al., 2022).

Furthermore, reproductive and developmental toxicity is increasingly becoming recognized as an important part of overall toxicology in order to evaluate variable toxicological risks between women and men (Ema et al., 2017). In the general population the major source of human exposure to reproductive disrupt compounds is by ingestion of food contaminated during production, processing, and packaging (Lyche et al., 2009).

Regarding the toxicological profile of PTSO, acute and subchronic (90 days) assays and a battery of genotoxicity assays have been performed *in vivo* (Llana-Ruiz-Cabello et al., 2015; Cascajosa-Lira et al., 2020). No signs of pathological alterations or DNA damage were found in all of them (Mellado-Garcia et al., 2016). As far as we know, the reproductive and developmental toxicity of PTSO has not been examined yet. In this regard, among other studies, a toxicity study which includes several generations of animals is recommended in order to evaluate the potential toxics effects of PTSO on the reproductive and endocrine systems.

Therefore, the objective of this work was to carry out, for the first time, an *in vivo* study using male and female mice at three dose levels to investigate the potential toxics effects of PTSO on the reproductive system taking into account fertility, genetic and endocrine endpoints based on OECD Guideline 416 (OECD, 2001). To achieve this global objective, organs related to reproduction (ovaries, uterus, epididymis, and testes) were weighed, and the body weight and brain ratios were calculated. Moreover, an analysis of the estrous cycle was performed. Sexual hormone levels (progesterone -P-, testosterone -T-, estradiol -E2-, follicular stimulating hormone –FSH-, and luteinizing hormone -LH-) were measured in serum by ELISA. And finally, alterations in the expression of genes related to hormone synthesis (*Star, Cyp11a1, Hsd3b1, Hsd17b12*), hormone receptors (*Ar, Esr1 and Esr2*), and gametogenesis (*Bpm-15, Gdf-9 and Dazl*) were investigated using Quantitative Real-Time Polymerase chain reaction (RT-qPCR).

2. Materials and methods

2.1. Test product, reagents, and kits

PTSO (purity 25%, carried in sepiolite) was purchased from DMC Research Center (Granada, Spain). The mice diets were prepared and made into pellets by Altromin Spezialfutter GmbH & Co. KG (Lage, Germany), by mixing the rodent feed, the solid support sepiolite, and the adequate amounts of PTSO to reach the desired doses. Reagents for RT-qPCR were supplied by Qiagen (Madrid, Spain) and Bio-Rad Laboratories (Hercules, CA, USA). ELISA kits for T and P were supplied by LDN (Nordhorn, Germany; catalog no: AR E−8000R and AR E−8700R, respectively), and for E2 by Biomatik (Ontario, Canada; catalog no: EKC40196). MILLIPLEX® Mouse Pituitary Magnetic Bead Panel for FSH, and LH was obtained from Millipore-Sigma-Aldrich (Madrid, Spain; catalog no: MPTMAG-49K). All other reagents were supplied by Sigma-Aldrich (Madrid, Spain).

2.2. Animals and housing conditions

A reproduction toxicity study was performed at the Central Service of Experimental Animals (SAE) of the University of Córdoba (Spain), based on the OECD Guideline 416 (OECD, 2001). Animals used in this work were CD1 (Swiss) mice, since this species was previously used by other authors to perform one- and two-generation studies (Tyl et al., 2008a, b, c). Although the rat is the preferred species for testing, mice were the species of choice in the present reproduction study due to several reasons: 1) a great number of animals are needed to perform this study; 2) mice are smaller than rats, which makes it easier the handling and housing; 3) the reduced cost of mice compared to rats, making them more accessible for studies with limited budgets requiring large amount of animals; 4) mice have a shorter reproductive cycle and a higher fertility rate than rats, allowing for faster and more efficient production of animal models. Mice were cared in agreement with the Directive for the protection of animals used for scientific purposes (Directive, 2010/63/UE, Decision 2020/569/UE and Real Decreto, 2018), and all procedures were authorized by the Ethical Animal Experimentation Committee of the University of Córdoba and by the Junta de Andalucía (project no. 26-06-2018-104).

CD1 (Swiss) mice (80 males and 80 females), supplied by Charles River laboratories (Kings, NY, USA), were approximately 5 weeks old at arrival at SAE, with average body weights (b.w.) of 36.68 ± 2.48 g for males, and 27.27 ± 1.88 g for females. They were maintained for 1 week for acclimation, with controlled conditions of hygiene, temperature of 22 ± 3 °C, relative humidity of 50–60%, and under a 12h light/dark cycle. Mice were fed with estrogen-free pellet composition (ROD14IRR, Altromin, Germany) and water *ad libitum*. Mice were randomly distributed into the control and three dose groups (20 mice/sex/dose group). During the premating period (10 weeks), mice were housed in cages (type IIL 365 × 205 × 140 mm) in groups of two animals of the same sex. After week 10, for the mating period, each female was placed with a single male from the same dose group. Following positive evidence of copulation by examination of the presence of sperm or vaginal plugs (1 week), mated females were single-caged, and males were humanely killed (anesthetized by isofluorane after being fasted overnight −18h- and euthanized with CO_2). Females continued with exposure to the test or control diet during the gestation (3 weeks), littering and lactation (2 weeks) periods, and continuing through to the day of sacrifice. A blood sample was taken previously to sacrifice in all mice, and organ samples were obtained during necropsy.

2.3. Animals' exposure

For the whole duration of the experiment (11 weeks for males and 16

weeks for females), the feed was prepared weekly, and the amount consumed by mice was calculated to set the different doses. 55 mg PTSO/kg body weight (b.w.), the maximum tolerable dose (MTD) (Llana-Ruiz-Cabello et al., 2015) was selected as the highest dose, and the descending doses were calculated by a 2-fold interval factor, as previously explained in a subchronic study performed in rats with PTSO (Cascajosa Lira et al., 2020). Therefore, three dose groups of 55, 28 and 14 mg PTSO/kg b.w./day, and a control group only fed with an estrogen-free laboratory diet was also established. To reach these doses, the concentrations of PTSO in the feed had to be 320.8, 163.3 and 81.6 mg PTSO/kg feed, respectively.

2.4. Organ weights and ratios

Following sacrifice of mice, the uterus, ovaries, testes, and epididymis were removed and weighed from all the assayed animals. Organ weights, organ weights to terminal body weight ratio, and organ weights to brain weight ratio were calculated for each group and sex.

2.5. Estrous cycle analysis

Vaginal smears were collected for 5 days during the week before the mating period. The vagina of the restrained mice was inserted by a cotton-tipped swab, gently turned and rolled in the vaginal wall and then removed. After transferring cells to a dry glass slide by rolling the swab, it was air dried, and then the Diff-Quik stain is performed. The slides were covered and viewed immediately at 10x and 40x magnification under bright field illumination. Based on the presence or absence of nucleated epithelial cells, cornified epithelial and leukocytes, the phase of the estrous cycle was established (Felicio et al., 1984).

2.6. Serum sex hormones measurement

Blood was collected from mice hearts, after being fasted overnight and anesthetized, and the serum was obtained by centrifugation. Serum levels of P, T, E2, FSH, and LH were measured by the hormone specific kits mentioned in section 2.1, following the manufacturer's instructions and according to Casas-Rodríguez et al. (2023). A microplate spectrophotometer (Tecan Infinite M200, Grödig, Austria) was used to read the absorbance at 450 nm in the case of P, T, and E2. For FSH and LH using the MILLIPLEX®, due to the special care needed to keep the magnetic beads in the plate, a Bio-Plex® Handheld Magnetic Washer (catalog no: 171020100) was used for each washing step, using MAGPIX® Drive Fluid PLUS (Cat. No. 40–50030), as described in the kit, and the plate was read on a Bio-Plex® 200 Multiplex system (Bio-Rad Laboratories Inc., USA) from the Biology Service of the Centro de Investigación, Tecnología e Innovación (CITIUS) of the University of Seville. For readings with the Bio-Plex® 200, the following settings were set: Reporter gain: low PMT; DD Gates: 5000 (low) and 25,000 (high); Events: 50, per bead. Bio-Plex Manager™ version 4.1.1 software was used for data analysis.

2.7. Extraction of RNA from testes and ovaries and reverse transcription

The RNeasy Lipid Tissue Mini Kit™ (catalog no: 74804, Qiagen, Madrid, Spain) was used for extraction and purification of total RNA from the testes and ovaries of mice, following the instructions from the manufacturer. Samples were also submitted to an additional on-column DNase digestion during the RNA purification process, using the RNAse-free DNase set (catalog no: 79254), and according to Diez-Quijada et al. (2022).

2.8. Gene expression analysis by quantitative real-time PCR

Levels of mRNA of genes related to reproductive functions were measured in the reproductive organs from mice by RT-PCR: *Cyp11a1*, *Hsd3b1*, *Hsd17b12*, *Star*, *Ar*, *Er1*, *Esr2*, *Bpm15* and *Gdf9* (ovaries), and *Cyp11a1*, *Hsd3b1*, *Hsd17b12*, *Star*, *Ar*, *Er1*, *Esr2*, and *Dazl* (testes). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was employed as an internal control. The diluted cDNA (1:2) was amplified by the PCR in a 384-well plate, in 10 μL final reaction volume, together with the iTaq universal probes Supermix (catalog no: 1725134) and the primePCR probe assay for the corresponding genes. LightCycler®480 (Roche, Berlin, Germany) was used for amplification: 95 °C (2 min), 50 cycles of 95 °C (5 s), and 60 °C (30 s), and the data collected were analyzed with software, using the threshold cycle (Ct) values to calculate the RNA concentration in the samples. For the treated samples, $2^{-\Delta\Delta CT}$ symbolizes the fold change in the expression of mRNA with respect to the housekeeping gene *GAPDH* (internal control) and to the calibrator (the untreated control) (Livak and Schmittgen, 2001).

2.9. Molecular docking analysis of PTSO with estrogen and androgen receptor

PTSO structure was obtained from ChemDraw (version 22.0) and energy-minimized using the minimization protocol of PyMOL. The obtained final conformation at energy minima was selected as the initial conformation for docking analysis. The automated docking method was applied to estimate the appropriate complex structure between PTSO and estrogen receptor (ER). Four different 3D crystalized structures of ER were downloaded from Protein Data Bank: 1ERE, 1ERR, 3ERD and 3ERT (Nose et al., 2009), and only one structure for androgen receptor (AR). In order to use an appropriate receptor molecule for the docking calculation, missing hydrogen atoms were added with PyMOL (version 2.5). The bound ligands and water molecules were removed from the 3D structures.

For the docking calculation, the AutoDock 3.0 software was employed, leaving enough space in the grid box to allow the docking between the ligands and the receptors (80 × 80 × 80 Å). Docking with the ER was completed with the prediction of the possible agonist or antagonist activity for PTSO, using 17-β-estradiol (E2) as control for the agonist activity, and 4-hydroxytamoxifen for the antagonist activity. To set a parameter to determinate the agonist/antagonist specificity, the factor *Cpf* (conformation preference factor) = $\Delta\Delta G$ was defined according to Nose et al. (2009):

$Cpf = \Delta G$ (agonist conformation) – ΔG (antagonist conformation).

Where ΔG (agonist conformation) is the average ΔG value from the docking calculations using 1ERE and 3ERD, and ΔG (antagonist conformation) is the average ΔG value from the docking calculation using antagonist bound (1ERR and 3ERT).

In the case of AR docking, it is not possible the agonist/antagonist docking screening method because the structural changes of this receptor when facing an agonist or an antagonist have not been characterized (Singam et al., 2019).

2.10. Statistics

Data were reported as mean ± standard deviation (SD). Statistical analyses were performed with GraphPad Prism 9 software (GraphPad Software Inc., La Jolla, CA, USA) by one-way Analysis of Variance (ANOVA), using Kolmogorov-Smirnov test for normality assumption. When statistically significant, comparisons were made with Tukey-Kramer Multiple Comparisons Test or with Kruskal-Wallis test followed by Dunn's multiple Comparison Tests, if non-normality was found. Differences were considered significant from $p < 0.05$.

3. Results and discussion

With the increasing use of natural bioactive compounds in the agrifood industry, their safety assessment is becoming more essential. Moreover, research on xenobiotics, which can alter the endocrine system of humans, vertebrates, and invertebrates, has experienced a

Table 1
Absolute organ weights (g), relative organ weight/body weights (%), and relative organ weight/brain weights (%) of the reproductive organs of male and female mice values after exposure to PTSO (0, 14, 28 and 55 mg/kg b.w./day).

	Dose groups (mg PTSO/kg b.w./day)			
	0	14	28	55
Male				
Left testis (g)	0.14 ± 0.02	0.14 ± 0.02	0.14 ± 0.02	0.14 ± 0.02
Right testis (g)	0.14 ± 0.02	0.14 ± 0.03	0.14 ± 0.02	0.13 ± 0.02
Left epididymis (g)	0.08 ± 0.01	0.07 ± 0.02	0.07 ± 0.01	0.07 ± 0.01
Right epididymis (g)	0.06 ± 0.01	0.07 ± 0.02	0.06 ± 0.01	0.07 ± 0.02
Left testis/b.w. ratio (%)	0.28 ± 0.02	0.25 ± 0.04	0.25 ± 0.05	0.27 ± 0.05
Right testis/b.w. ratio (%)	0.28 ± 0.04	0.26 ± 0.06	0.25 ± 0.05	0.27 ± 0.06
Left epididymis/b.w. ratio (%)	0.14 ± 0.01	0.13 ± 0.02	0.13 ± 0.01	0.14 ± 0.04
Right epididymis/b.w. ratio (%)	0.13 ± 0.03	0.12 ± 0.03	0.11 ± 0.02	0.13 ± 0.04
Left testis/brain ratio (%)	29.28 ± 3.08	27.51 ± 3.04	28.69 ± 4.03	27.91 ± 4.68
Right testis/brain ratio (%)	28.63 ± 4.04	28.13 ± 6.30	28.53 ± 3.99	27.06 ± 4.42
Left epididymis/brain ratio (%)	15.30 ± 2.63	14.32 ± 3.25	12.20 ± 4.08	14.25 ± 2.38
Right epididymis/brain ratio (%)	12.44 ± 2.01	13.92 ± 3.89	12.14 ± 2.13	13.28 ± 2.83
Female				
Uterus (g)	0.25 ± 0.11	0.30 ± 0.16	0.22 ± 0.07	0.26 ± 0.10
Left ovary (g)	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
Right ovary (g)	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.00	0.02 ± 0.01
Uterus/b.w. ratio (%)	0.62 ± 0.27	0.59 ± 0.23	0.54 ± 0.18	0.65 ± 0.25
Left ovary/b.w. ratio (%)	0.05 ± 0.01	0.06 ± 0.04	0.05 ± 0.01	0.05 ± 0.02
Right ovary/b.w. ratio (%)	0.05 ± 0.01	0.06 ± 0.02	0.05 ± 0.01	0.06 ± 0.03
Uterus/brain ratio (%)	48.51 ± 21.87	53.98 ± 22.94	45.08 ± 15.46	52.94 ± 21.10
Left ovary/brain ratio (%)	4.33 ± 0.83	4.67 ± 1.68	4.14 ± 1.02	4.45 ± 1.57
Right ovary/brain ratio (%)	4.10 ± 1.23	4.45 ± 1.15	4.12 ± 0.96	4.32 ± 1.07

notable surge recently (Cunha et al., 2022; Macedo et al., 2023). Although this aspect was assessed previously in a 90-day oral subchronic toxicity study of PTSO (Cascajosa-Lira et al., 2020), its reproductive toxicity has never been evaluated before. In this work, the effects of this compound on the reproductive system are studied for the first time in mice (both sexes) exposed orally to PTSO for 11 (males) or 16 (females) weeks to 14, 28, or 55 mg PTSO/kg b.w./day. The results have covered a wide range of aspects such as organ weights and ratios, estrous cycle analysis, serum hormone levels, genes related to reproduction functions, as well as *in silico* predictions by molecular docking analysis.

3.1. Organ weights and ratios

No mortality was recorded during the experiment, and no other clinical observations were noted. The weight of endocrine organs, such as ovaries and testes, is used as an indicator of possible effects of exposure to endocrine disruptors in animal studies (OECD 440, 2007b; OECD 441, 2009; Casas-Rodríguez et al., 2023). Results obtained for absolute organ weights (g), organ weights to terminal body weight ratio (%), and organ weights to brain weight ratio (%) are presented in Table 1. No changes in any of those parameters were observed during the study, in agreement with the results obtained by Cascajosa-Lira et al. (2020) in rats subchronically exposed to PTSO for 13 weeks.

3.2. Estrous cycle

Along the experiment, there were no significant major changes in the estrous cycle between female mice exposed to PTSO and the control ones. As cell types of vaginal smears varied periodically with the estrogen levels of female mice, the determination of the estrous cycle phase was established from the cell type observed (Fig. 1). In this sense, in the proestrus stage, the nucleated epithelial cells are the predominant ones; the cornified squamous epithelial cells are mainly detected at the estrus phase; leukocytes are predominant at the stage of metestrus; and all these three types of cells could be detected at diestrus. Increments of 10% and 5% in the incidence of extended anestrus were found in female mice exposed to 28 and 55 mg PTSO/kg b.w./day, respectively, compared to the control mice. Anestrus is the period of ovarian inactivity during which there is no ovulation or secretion of reproductive hormones. Apart from that, 5%, 25% and 15% of female mice treated with the above-mentioned doses, did not cycle (staying in diestrus phase); and 10% of female mice exposed to the highest dose of PTSO presented extended estrus phase. However, these differences are not dose-dependent, suggesting that they are more likely attributed to inter-individual variability rather than to the effect of PTSO. The cellular composition and cell ratio vary among individual rodents during the estrous cycle, especially during diestrus (Cora et al., 2015). In certain animals, a significant number of neutrophils and nucleated epithelial cells may be present, while in others, they may be scarce (Long and Evans, 1992; Cora et al., 2015).

3.3. Serum sex hormone levels

The serum levels of P, T, E2, FSH and LH for male and female mice are represented in Fig. 2. Progesterone levels show significant increases in male mice treated with lower doses of PTSO compared to the control. However, there is not a clear dose-dependent relation between P affectation and PTSO doses, since no significant differences were observed compared to the control at the highest tested dose of 55 mg/kg b.w./day. Moreover, this affectation could not be attributed to any changes in the expression of steroidogenic genes, as no alterations were found in male mice exposed to PTSO at any dose assayed in any of the genes studied. On the contrary, no significant alterations were detected in female mice in P serum concentration at any doses assayed.

Serum levels of T for males and females showed no significant differences with their respective control groups, and values found in all control and dose groups are in a normal range of concentrations for both sexes (Chapman et al., 1998; Fan et al., 2015; O'Hara et al., 2015; Schellino et al., 2016).

Estradiol serum levels showed no significant differences in male samples and their hormonal levels are within a normal range (Rao et al., 1982). However, even though female mice show a significant decrease of E2 at the lower doses in comparison with the control group, these serum levels are in a normal range considering the deviations that this hormone has depending on the phase of the estrous cycle (Barkley et al., 1979; Yi et al., 2022).

Follicular stimulating hormone serum levels for male and female mice show no significant differences compared to their respective control groups for both sexes. LH levels present significant decreases in male mice at the lower doses in comparison with the control group. However, once again, no dose-dependent effect was observed, as no significant alterations were detected at the highest tested dose. In addition, it is remarkable that these serum concentrations are within the normal range reported by other authors of 300–500 pcg/mL (O'Hara et al., 2015; Musicki et al., 2015). Likewise, LH levels of female mice showed a significant increase at 14 mg/kg b.w./day, in agreement with normal levels reported in female mice in the range 150–1300 pcg/mL (Czieselsky

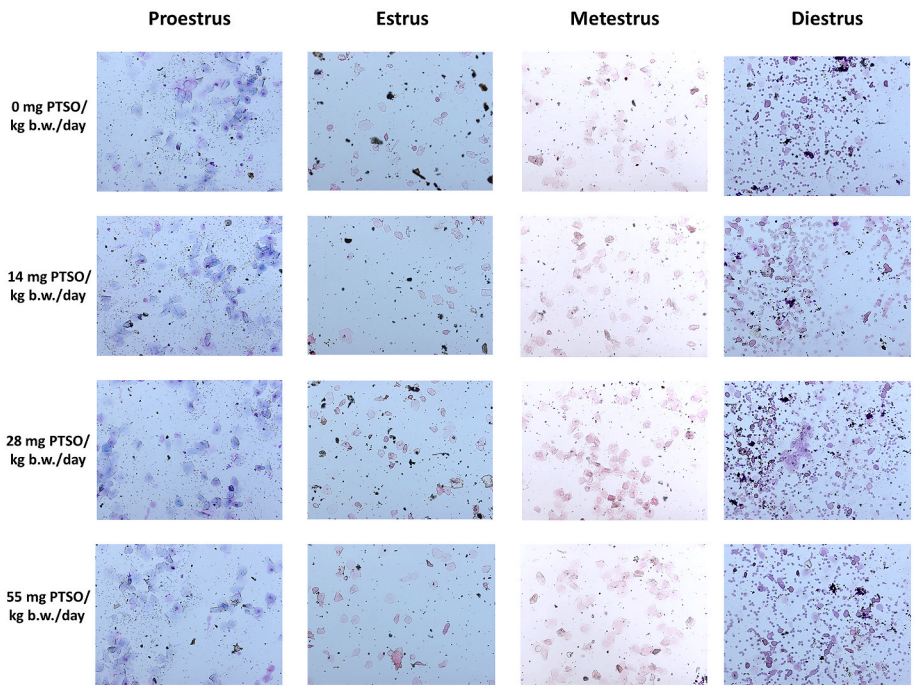

Fig. 1. Vaginal smears under light microscope from female mice at proestrus, estrus, metestrus, and diestrus stage. The proportion of epithelial cells, cornified cells, and leucocytes was used for determination of the estrous cycle phases.

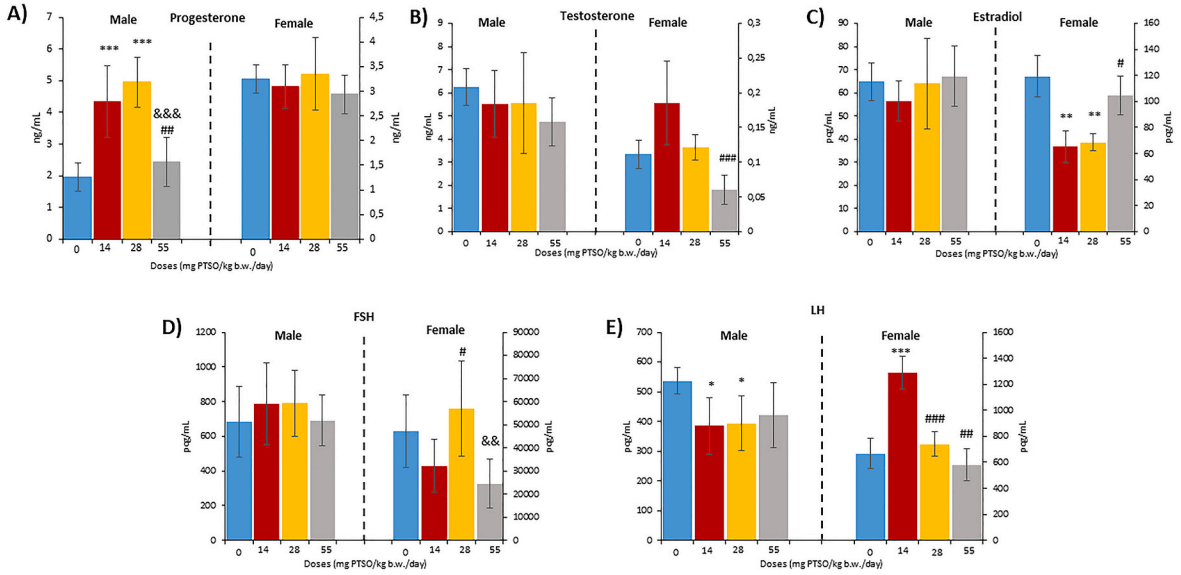

Fig. 2. Serum levels (ng/mL or pcg/mL) of sexual hormones: Progesterone (a), Testosterone (b), Estradiol (c), Follicle stimulating hormone (d), and Luteinizing hormone (e), in male mice (left axis) and female mice (right axis). Mean values (±SD) of N = 6 are calculated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). The coded symbols represent the following: *—statistical differences compared with the respective control group. #—statistical differences compared with 14 mg PTSO/kg b.w./day. &—statistical differences compared with 28 mg PTSO/kg b.w./day.

et al., 2016; Brehm et al., 2020).

3.4. Expression analysis of reproductive-related genes by quantitative real-time PCR

Fig. 3 displays the relative gene expression of the assayed genes in the testes and ovaries of mice exposed to PTSO. Genes related to four different endpoints were studied: steroidogenesis (*Star*, *Cyp11a1*, *Hsd3b1*, and *Hsd17b12*), hormone receptors (*Ar*, *Esr1*, and *Esr2*), spermatogenesis (*Dazl*) and oogenesis (*Bmp-15* and *Gfd-9*).

The effects of PTSO in gene expression concerning the reduction of oxidative stress and improvement of the immune response were demonstrated in previous studies. In this sense, PTSO induced a localized antioxidant response associated with Nrf2 signaling against *Citrobacter rodentium* (Zhu et al., 2022a) and induced significant changes in intestinal gene expression and pathways related to immune function and inflammation against *Trichurus muris* in mice (Zhu et al., 2022b). This pattern has also been previously detected in pigs, where the addition of PTSO to the diet attenuated the heightened immune response caused by *E. coli* infection through the reduction in transcriptional signaling

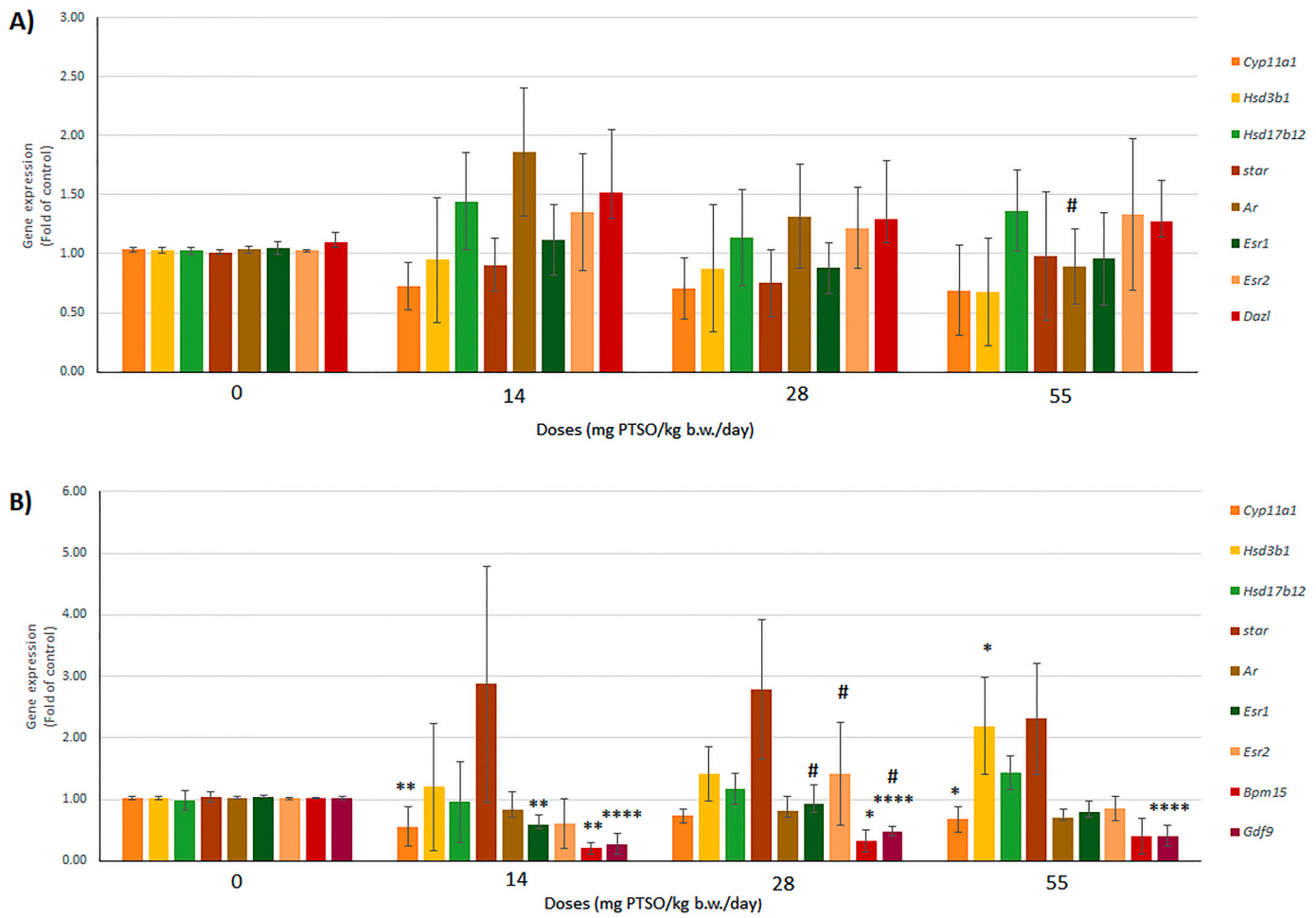

Fig. 3. *Cyp11a1, Hsd3b1, Hsd17b12, Star, Ar, Esr1, Esr2, Bpm-15, Gdf-9, Dazl* gene expression expressed as fold of control in the testes of male (A) and ovaries of female (B) mice in the different exposure scenarios (0, 14, 28, and 55 mg PTSO/kg b.w./day). Mean values (±SD) of N = 6 are calculated (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$). The coded symbols represent the following: *—statistical differences compared with the respective control group. #—statistical differences compared with 14 mg PTSO/kg b.w./day. *Cyp11a1*: cholesterol monooxygenase (side-chain-cleaving); *Hsd3b1*: 3beta-hydroxysteroid dehydrogenase/delta(5)-delta (4)isomerase; *Hsd17b12*: 17-beta hydroxysteroid dehydrogenase −12; *Star*: Steroidogenic Acute Regulatory Protein; *Ar*: Androgen receptor; *Esr1*: Estrogen receptor alpha; *Esr2*: Estrogen receptor beta; *Bpm-15*: Bone morphogenetic protein 15; *Gdf-9*: Growth differentiation factor-9; *Dazl*: Deleted in Azoospermia-Like.

pathway and antigen presentation (Liu et al., 2014a; Liu et al., 2014b). In the same way, feeding young broiler chickens with PTSO led to significant changes in the transcriptome of chicken intestinal lymphocytes, positively affecting immune and cardiovascular-related gene pathways and networks (Kim et al., 2013).

In our study, no differences were observed in any of the assayed genes in the testes of male mice at any dose assayed (Fig. 3A). Among all the studied genes, Dazl was only measured in the testes of male mice as it is related to spermatogenesis. Dazl is expressed in differentiated spermatogonia and spermatocytes in the pachytene stage (Ghorbaninejad et al., 2023). The suppression of Dazl in the mouse results in the loss of germ cells in male gonads (by reduced expression of germ cell markers, apoptosis, and aberrant chromatin structure) (Xu et al., 2013), as this gene is essential for the development and survival of germ cells (Hsien-An Pan et al., 2008).

By contrast, as shown in Fig. 3B, female mice showed alterations in steroidogenesis, hormone receptors and oogenesis genes after exposure to all the doses tested, especially to 14 mg PTSO/kg b.w./day. At the beginning of hormone synthesis, steroidogenic acute regulatory protein (*Star*) is responsible for transferring cholesterol through the mitochondrial membrane into the mitochondria (Lin et al., 2022). Afterwards, cholesterol is oxidized by mitochondrial cytochrome P450 oxidase (*Cyp11a1*) and converted into pregnenolone, later oxidized by *Hsd3b1* and *Hsd17b12*, resulting in the formation of P, T and E2 (Daoud et al., 2021). In the ovaries of female mice, significant downregulations were shown in Cyp11a1 expression at 14 and 55 mg PTSO/kg b.w./day compared with the respective control group ($p < 0.01$ and $p < 0.05$, respectively). *Cyp11a1* (cholesterol side-chain cleavage enzyme), is in charge of oxidizing cholesterol to pregnenolone, a precursor hormone in the steroidogenesis pathway. The levels of downstream hormones such as E2, P and T could be decreased as a result of the downregulation of *Cyp11a1* (Gill et al., 2021). Nevertheless, it is important to note that the downregulation of these genes does not necessarily imply a reduction in enzymatic activity. Factors such as post-transcriptional regulation, mRNA stability, translational control, and protein turnover rates can contribute to variations between mRNA expression and enzymatic protein levels (Tian et al., 2004). Therefore, while certain genes involved in steroid hormone synthesis may experience downregulation, the observed improvements in lipid metabolism through the reduction of total and LDL cholesterol without affecting HDL cholesterol levels with PTSO suggest that downregulation does not automatically result in decreased cholesterol and steroid hormone production (Vezza et al., 2021). Conversely, another steroidogenic gene, *Hsd3b1*, was significantly upregulated in the ovaries of mice only at the highest dose compared with the control ($p < 0.05$), suggesting a modulatory effect of gene expression. *Hsd3b1* is a gene that codes for a 3β-hydroxysteroid

Table 2
Estimated binding energies obtained by docking of different agonists and antagonists, and PTSO with the activated or inactivated conformations of ER.

	Estimated free energy of binding (kcal/mol)						ΔG[a]	$\Delta\Delta G$ = Cpf[b]
	Agonist-bound receptor			Antagonist-bound receptor				
	1ERE	3ERD	Mean	1ERR	3ERT	Mean		
Agonist								
Estradiol (E2)	**−10.5**	−7.8	−9.15	−9	−7.5	−8.25	−8.7	−0.9
Diethylstilbestrol	−5	**−6.6**	−5.8	−5.2	−6.2	−5.7	−5.8	−0.1
Antagonist								
Raloxifene	−6	−7.8	−6.9	−6.5	**−8.1**	−7.3	−7.1	0.4
4-hydroxitamoxifen	−5.7	−6.4	−6.05	**−9.1**	−7.4	−8.25	−7.2	2.2
PTSO	−4.4	−4.4	−4.4	−3.3	−3.2	−3.25	−3.8	−1.15

[a] ΔG: average of binding energies from four docking calculations using 1ERE, 3ERD, 1ERR, and 3ERT.
[b] $\Delta\Delta G$ = Cpf (conformation preference factor): difference between average energies from the calculations used by agonist-bound receptor and antagonist bound receptor.

dehydrogenase, an enzyme that catalyzes the synthesis of P from pregnenolone (Simard et al., 2005).

Expression of Hsd17b12 did not show any significant changes in ovaries at the PTSO doses assayed in the present work. *Hsd17b12* encodes the 17-beta-hydroxysteroid dehydrogenase that converts P into E2 in ovarian tissue. The physiological role of *Hsd17b12* has given rise to controversy, as some suggested its function in the E1 to E2 conversion, whereas others supported that the enzyme is involved in the synthesis of arachidonic acid (Kemiläinen et al., 2016). It is essential for normal ovarian function, helping to regulate the estrus cycle, oogenesis, meiosis and ovulation. Kemiläinen et al. (2016) found the expression level of *Hsd17b12* varied during the different estrus cycle stages, detecting the highest expression at the diestrous and pseudopregnant ovaries. These authors indicated that the decline in the expression of *Hsd17b12* in mice ovaries did not result in lower levels of sex steroids, suggesting that *Hsd17b12* was not essential for ovarian steroid synthesis.

Concerning the expression of genes related to hormone receptors, a significant reduction in the expression of *Esr1* was observed only after exposure to the lowest dose of 14 mg PTSO/kg b.w./day ($p < 0.01$). By contrast, no significant differences in the expression of *Esr2* or *Ar* were observed in comparison to the control group. Recent observations have shown that normal folliculogenesis requires AR-mediated androgen action (Rodríguez et al., 2010). Likewise, ER is required for fertility in both sexes, even for spermatogenesis, sperm function, and mating performance (Eddy et al., 1996).

Significant downregulations were also observed for *Bpm15*

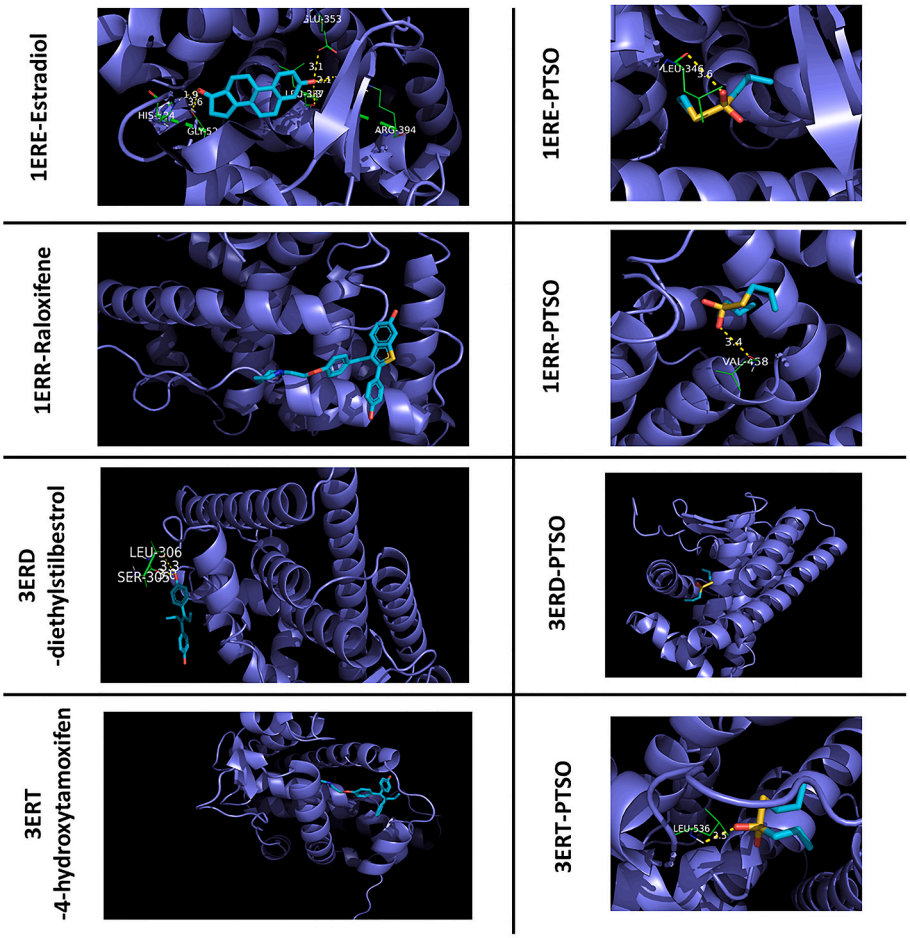

Fig. 4. Results of the docking calculations for the four different ER conformations (in purple: 1ERE, 1ERR, 3ERD, and 3ERT) with Estradiol, Raloxifene, diethylstrilbestrol, and 4-hydroxytamixifen, as well as with PTSO. Amino acid residues (green lines) are docked with the ligands (blue) by hydrogen bonding (doted yellow lines, and the measured distance in Angstrom), by the oxygen atom of the ligand, represented in red. In the ligand structure, sulfur is represented in yellow, and nitrogen in dark blue.

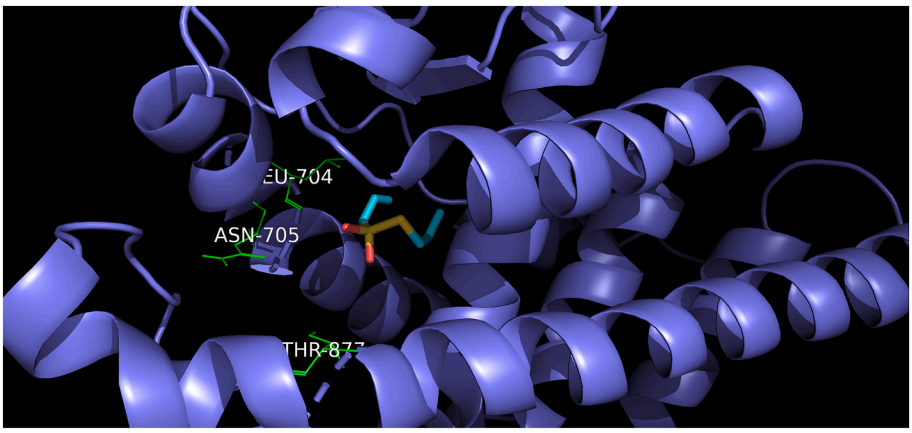

Fig. 5. Results of the docking between PTSO and the Androgen receptor (AR, in purple). The most relevant amino acid residues (green lines) are shown but not docked with PTSO.

expression at 14 and 28 mg PTSO/kg b.w./day ($p < 0.01$ and $p < 0.05$, respectively), but not at 55 mg PTSO/kg b.w./day, and for *Gdf9* expression for all doses assayed ($p < 0.0001$). *Bmp15* and *Gdf9* are involved in the development and function of the ovary and uterus (Jefferson et al., 2002; Fernandez et al., 2016; Gao et al., 2018). While female mice with deficient *Gdf9* could be infertile, blocking folliculogenesis at the stage of primary follicles (Belli and Shimasaki, 2018), subfertile female mice have resulted from *Bmp15*-deficient specimens, with a lower litter size and a lower number of litters per month, indicating a less essential role for *Bpm15* in mouse reproductive function compared with *Gdf9* (Takahashi et al., 2012). According to Belli and Shimasaki (2018), the absence of *Gdf9* induces higher serum levels of LH as compared to control. This is in line with our results, which show a decreased *Gdf9* expression together with higher LH levels in female mice exposed to the same PTSO dose (14 mg/kg b.w./day). However, despite the lack of dose-dependency in this response, the results reported by other authors who observed the modulation of fertility-related genes following treatment with *Allium* extract for the alleviation of polycystic ovary syndrome suggest the potential influence of these compounds in fertility regulation (Lee et al., 2018; Falahatian et al., 2022). Moreover, previous studies have shown that the dietary supplementation of PTSO in laying hens led to increased egg size and number suggesting an improvement of fertility (Abad et al., 2020; Sánchez et al., 2020). It is important to note that the regulation of fertility and follicular development involves a complex interplay of various mechanisms, and the downregulation of specific genes alone may not fully elucidate the overall impact on reproductive function. Additional studies are needed to explore the comprehensive effects of PTSO and its potential interactions with other molecular pathways involved in folliculogenesis, hormone regulation, and fertility.

Moreover, in the present work, the expression of genes related to different physiological processes related to reproduction (growth factors, hormone synthesis or hormone receptors) were studied. However, other authors in similar studies have also included genes related to tumor induction (bcl2, c-kit), more receptors (fshr, lhcgr), or genes related to the antioxidant activity (sod1, sod2, cat) in reproductive organs (Dostalova et al., 2020). Future research to investigate potential changes in these genes related to reproduction could be carried out.

3.5. Molecular docking analysis of PTSO with estrogen and androgen receptors

In order to perform a molecular docking analysis with ER, firstly, the agonist/antagonist differential screening method was validated for known ligands of ER. This methodology predicts the agonist or antagonist activity of small molecules using their binding energy which was calculated by AutoDock Vina 3.0. The 3D structures of ER with no ligands were named: 1ERE, 3ERD, for the activated conformation, and 1ERR and 3ERT for the inactivated conformation. These structures were used as templates to evaluate the ability to bind with their natural ligand or PTSO, and the resulting binding energies are shown in Table 2. When agonist molecules were coupled to the activated conformation of the ER, the average binding energy was lower than that obtained when docking with receptors in the inactivated conformation. In the case of antagonist molecules, the average binding energy obtained in docking with receptors in the inactivated conformation was lower, showing that agonists are more stable in activated conformations and antagonists in inactivated conformations.

The average binding energy of PTSO in the activated conformation was −4.4 kcal/mol (Table 2), while in the inactivated conformation was −3.25 kcal/mol. The difference between these average energies was −1.15 kcal/mol indicating that PTSO is 1.15 kcal/mol more stable in the activated conformation than in the inactivated conformation. However, the binding energies of PTSO and the ER are too high compared to those obtained after binding agonists or antagonists, which could indicate the inability of PTSO to interact with this receptor.

Fig. 4 shows the polar interactions between the ER, their more stable agonist or antagonist and PTSO. When E2 was docked with the activated conformation of estrogen receptor 1ERE, it was hydrogen bonded to Arg394, Glu353, His524, and Gly521. By contrast, when PTSO bounded to this receptor, no polar bonds were produced with these amino acids, but with Leu346. When Raloxifene was docked with the inactivated conformation of estrogen receptor 1ERR, no hydrogen binding was detected. However, when PTSO bounded to this receptor, one hydrogen binding was found with Val458. When Diethylstilbestrol was docked with the activated conformation of estrogen receptor 3ERD, it was hydrogen bonded with ser305 and Leu306, but when PTSO bounded to this receptor, no hydrogen binding was found. When 4-hydroxytamoxifen was docked with the inactivated conformation of estrogen receptor 3ERT, it was not hydrogen bonded, but PTSO was hydrogen bonded to 3ERT by Leu536. In conclusion, the interactions presented by the tested PTSO molecule do not have common polar interactions between the ER and its preferred agonists or antagonists in their different conformations.

For AR, only one conformation was used since it seems that agonist or antagonist effect is not related to conformational changes of this receptor. This single conformation has been utilized for AR due to the lack of evidence suggesting that the antagonist effect is connected to conformational changes in this receptor. To predict the binding poses and affinities of screened ligands through molecular docking analysis, a representative conformation of the receptor is necessary. However, up to date, there is no available crystal structure of the AR receptor bound to an antagonist (Wahl and Smiesko, 2018). This fact is one of the

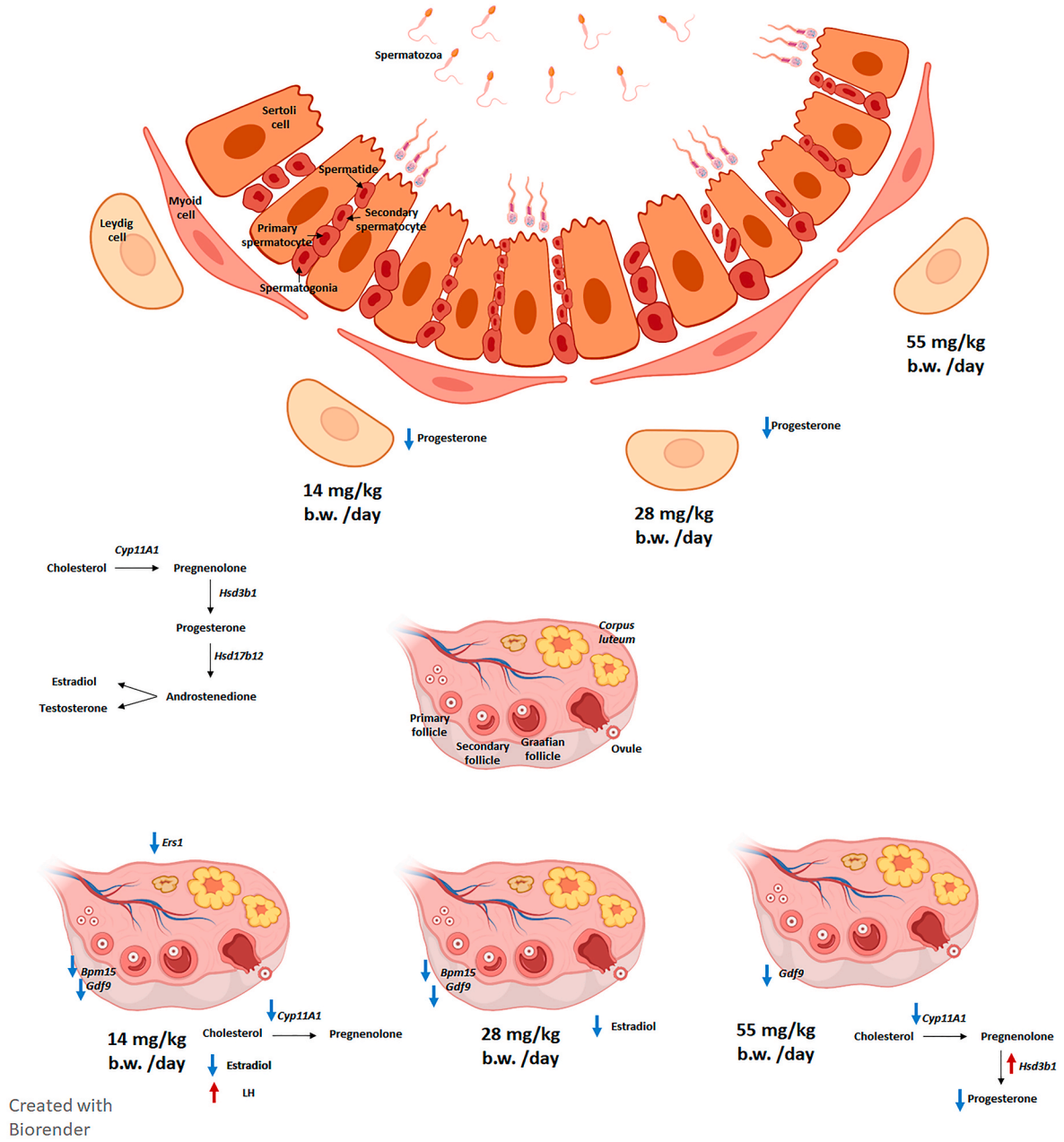

Fig. 6. Summary of the results obtained from gene expression and serum hormone levels in mice exposed to different doses of PTSO (0, 14, 28 and 55 mg PTSO/kg b. w./day). Testicular and ovary tissues, with different cell population present in testes and ovaries are depicted, showing the alterations found in them.

limitations of molecular docking analysis to predict potential toxicological effects related to androgen disruption. The mechanism by which an antagonist becomes an agonist or *vice-versa* is still elusive (Bisson et al., 2008; Singam et al., 2019). For example, hydroxyflutamide can act as an agonist or antagonist depending on the concentration (Wilding et al., 1989; Kemppainen and Wilson, 1996). However, some authors have demonstrated that potential agonists present hydrogen bonds with Thr877 and potential antagonists or partial agonists present hydrogen bonds with Leu704 and Asn705 (Singam et al., 2019). Fig. 5 shows the docking between PTSO and the AR, as well as the location of the relevant amino acids mentioned above. However, PTSO was not hydrogen bounded with any amino acid of this receptor and the estimated binding energy was −4.5 kcal/mol. Therefore, as in the case of the estrogen receptor, the interaction between PTSO and the androgen receptor could not be possible.

In both cases, for estrogen and androgen receptors, experimental studies are needed to validate these findings, following OCDE guidelines (OECD 455 and 458); as far as we know, no assays have been performed.

The most relevant findings observed in the present work are summarized in Fig. 6.

4. Conclusions

The present work shows for the first time a reproductive and endocrine toxicological study of PTSO. No significant changes were detected in the weight of the organs and their ratios (with respect to the body weight or the brain), as well as in the estrous cycle analysis. Serum levels of the studied hormones were not altered by exposure to PTSO, except E2 in females and LH in both sexes, although they stayed within normal ranges, and P in males. In terms of genetic endpoints, females exhibit a higher incidence of gene expression changes related to female reproduction, specifically in genes such as *Cyp11a1*, *Hsd3b1*, *Esr1*, *Bpm15*, and

Gdf9, although they do not translate into macroscopic changes in organs. In contrast, no genetic alterations were observed in male mice. The docking results showed the impossibility of stablishing stable polar binding to ER in their agonist or antagonist conformation as well as to the AR. These obtained results demonstrate the safety profile of PTSO for different agrifood applications and its consequences in the environment.

Author contributions

Conceptualization, A.M.C. and S.P.; methodology, A.C.-L., N.A.-S., A. M., R.M.-S., A.M.C, and S.P.; software, A.C.-L., R.G.G.; formal analysis, A.C.-L., R.G.G., A.J. A.M.; investigation, A.C.-L., R.G.G., A.B.A., M.A.A.-C., N.A.-S., R.M.-S., A.M., A.J., A.M.C. and S.P; resources, A.B.A., R.M.-S., N.A.-S., A.M., A.J., A.M.C. and S.P.; data curation, A.C.-L., R.G.G., and N.A.-S.; writing—original draft preparation, A.C.-L., R.G.G., N.A.-S., A.M., and M.A.A.-C.; writing—review and editing, A.J., A.M.C., S.P., and A.B.A.; visualization, A.C.-L., R.G.G.; supervision, A.J., A.M.C. and S.P.; project administration, A.M.C. and S.P.; funding acquisition, A.M.C. and S.P. All authors have read and agreed to the published version of the manuscript.

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All animals received human care in agreement with the Directive for the protection of animals used for scientific purposes (Directive, 2010/63/UE, Decision 2020/569/UE and RD 1386/2018), and all procedures were authorized by the Ethical Animal Experimentation Committee of the University of Córdoba and by the Junta de Andalucía (project no. 26-06-2018-104).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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