The assessment of the intergenerational effects induced by persistent pollutants on fertility

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INTERNSHIP REPORT

The assessment of the intergenerational effects induced by persistent pollutants on fertility

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ABSTRACT

Environmental pollutants, including Cadmium (Cd), Ochratoxin A (OTA), and Perfluorooctanoic acid (PFOA), pose significant threats to reproductive health and genetic stability. This study investigates the effects of these toxicants on mice's reproductive system, focusing on the directly exposed parental generation (F0) and their offspring (F1).

Our findings reveal that exposure to Cd, OTA, and PFOA results in significant reproductive toxicity, including reduced spermatozoa numbers and disrupted testosterone levels and gene expression. In the F1 generation, we observed compensatory changes in gene expression related to spermatogenesis and hormone signaling, particularly following PFOA exposure. Epigenetic analyses showed that DNA methylation in sperm of F0 mice correlates with gene expression changes in F1 testis, highlighting the role of epigenetic mechanisms in intergenerational effects.

These results revealed the complex interplay between environmental pollutants, epigenetic modifications, and reproductive health across generations, emphasizing the need for stringent regulatory measures and further research to mitigate these risks.

Keywords: Cadmium, Ochratoxin A, Perfluorooctanoic acid, sperm DNA methylation, spermatogenesis, intergenerational inheritance, hormone signaling pathway

RÉSUMÉ

Les polluants environnementaux, notamment le cadmium (Cd), l'ochratoxine A (OTA) et l'acide perfluorooctanoïque (PFOA), constituent des menaces importantes pour la santé reproductive et la stabilité génétique. Cette étude examine les effets de ces substances toxiques sur le système reproducteur des souris, en se concentrant sur la génération parentale directement exposée (F0) et sur leur progéniture (F1).

Nos résultats révèlent que l'exposition au Cd, à l'OTA et au PFOA entraîne une toxicité importante pour la reproduction, notamment une réduction du nombre de spermatozoïdes et une perturbation des niveaux de testostérone et de l'expression des gènes. Dans la génération F1, nous avons observé des changements compensatoires dans l'expression des gènes liés à la spermatogenèse et à la signalisation hormonale, en particulier après l'exposition à l'OTA et au PFOA. Les analyses épigénétiques ont montré que la méthylation de l'ADN dans le sperme des souris F0 est en corrélation avec les changements d'expression génique dans les testicules de la génération F1, soulignant le rôle des mécanismes épigénétiques dans les effets intergénérationnels.

Ces résultats ont révélé l'interaction complexe entre les polluants environnementaux, les modifications épigénétiques et la santé reproductive d'une génération à l'autre. Ils soulignent le besoin urgent de réglementations strictes et de recherches supplémentaires pour réduire ces risques.

Mots clés: Cadmium, Ochratoxine A, Acide Perfluorooctanoïque, méthylation de l'ADN du sperme, spermatogenèse, héritage intergénérationnel, voie de signalisation hormonale.

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1. INTRODUCTION

1.1. Spermatogenesis

Spermatogenesis is a process by which haploid spermatozoa are produced from germ cells in the seminiferous tubules of the testis (de Kretser et al., 1998). This process includes DNA replication and two rounds of meiotic cell division, in which a single diploid cell gives rise to 4 haploid cells. The germinal layer of the seminiferous tubules, known as the seminiferous epithelium, contains spermatogenic cells and Sertoli cells (Dettin et al., 2003). Sertoli cells surround the proliferating and differentiating germ cells, forming pockets around these cells, supplying nutrients, and phagocytosing excess spermatid cytoplasm that is not required for spermatozoa formation (Griswold, 1995).

As shown in **Figure 1.**, spermatogenesis begins with the mitotic division of spermatogonial cells located next to the basal lamina. This division results in two types of cells – type A spermatogonia, which either continue to divide to maintain a reserve of spermatogonia or remain dormant; and type B spermatogonia, which undergo mitotic division and serve as differentiating precursors of primary spermatocytes (Dym et al., 2009). Tetraploid primary spermatocytes proceed through the first meiotic division, producing two secondary spermatocytes. This division is crucial as it reduces the chromosome number by half, resulting in the formation of diploid secondary spermatocytes, which then undergo the second meiotic division forming 4 haploid spermatids. This haploid state is essential for the correct number of chromosomes in the offspring. In the final stage of spermatogenesis, these mature, haploid spermatids metamorphose into fully formed spermatozoa (Dali et al, 2024). The process culminates as these spermatozoa detach from the Sertoli cells, which have nourished and supported them through their maturation, and move into the lumen of the seminiferous tubules. Once matured, the sperm is transported through the long seminiferous tubules and stored in the epididymis until it is ready to be expelled from the male body via ejaculation. It is suggested that spermatozoa and oocytes might play a role in intergenerational inheritance, a complex event where acquired phenotypic alterations could be passed on to subsequent generations.

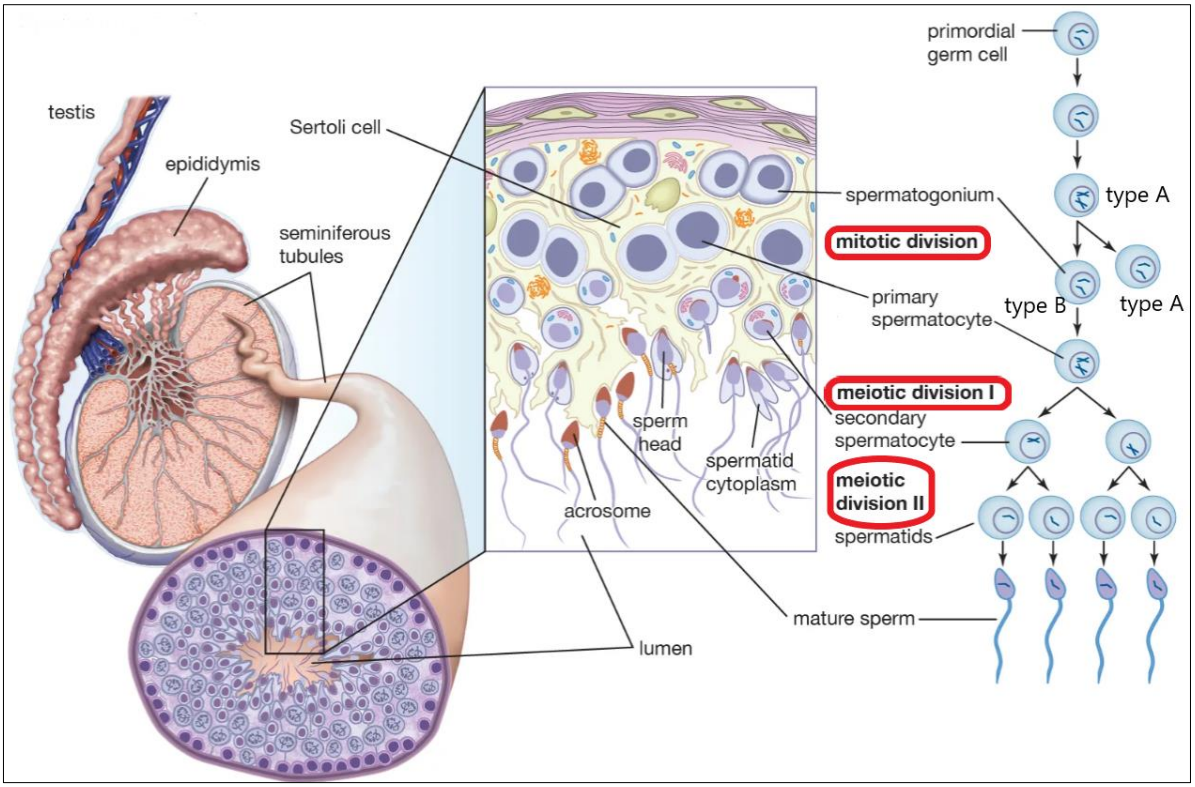

Figure 1. Scheme of spermatogenesis and its main stages: from multiplication via mitosis to maturation to spermatozoa (on the right). The path spermatozoa take through seminiferous tubules to the epididymis is shown on the left. (Encyclopedia Britannica, 2013).

1.2. Environmental Pollutants and Public Health

Environmental pollutants represent a growing global concern because of their extensive presence and profound impact on human health. These pollutants include a broad range of chemical, biological, and physical agents released into the environment through industrial processes, agricultural activities, urban development, and improper waste disposal. Once released, they can contaminate the air, water, and soil, creating pathways for human exposure through inhalation, ingestion, or direct contact (Jones et de Voogt, 1999). The interaction between humans and these environmental contaminants can lead to adverse health outcomes. The severity and type of health effects depend largely on the pollutant's nature, exposure level and duration, and individual susceptibility (Sharpe, 2010).

Among the most troubling impacts of environmental pollutants is their effect on reproductive health. Specifically, certain pollutants have been identified as endocrine disruptors, which can interfere with the hormonal systems that regulate reproductive functions. This disruption can manifest distinctly in males, affecting sperm quality, quantity, and overall reproductive capability (Foster, 2008). Lately, persistent pollutants (PPs) have become a primary focus of research in this field. The recent European program HBM4EU (Human Biomonitoring for Europe) has prioritized several substances for urgent risk assessment, including Cadmium (Cd), Ochratoxin A (OTA), and Perfluorooctanoic acid (PFOA). Understanding the specific pathways and mechanisms through which these 3 pollutants exert their

effects is critical for developing effective interventions and policies to mitigate their impact and protect human health.

Cadmium (Cd), a heavy metal identified as a persistent pollutant (PP), exhibits varied environmental concentrations originating from both natural sources and anthropogenic activities. Naturally, cadmium is released through the erosion of cadmium-rich rocks, volcanic activities, and forest fires. However, its anthropogenic release is significantly influenced by its industrial applications, including its use in automotive radiators, alkaline batteries, mining operations, and as a coloring pigment and stabilizer in plastics. This usage leads to cadmium's release into the environment through routes such as sewage sludge and improper waste disposal. In agriculture, cadmium is a component of some fertilizers, which can contaminate the soil and eventually make its way into the crops that humans can consume (WHO, 2010).

This metal spreads through the air, water, and soil. Previous studies show that rural parts of Europe have 10-100 times lower concentrations of cadmium in the air compared to urban regions. In natural water bodies like rivers and lakes, cadmium levels are relatively low. However, drinking water might get contaminated through galvanized pipes, water heaters, or if cadmium-containing waste leaks into the groundwater. As for soil, cadmium levels are low in the areas untouched by pollution. However, in agricultural regions, certain fertilizers can lead to higher cadmium concentrations (U.S. Geological Survey, Yearly Reports).

Cadmium is known to have numerous adverse effects on human health, including reduced fertility and issues with the male reproductive system, renal dysfunction, and adverse effects on the brain. It is also classified as a carcinogen (HBM4EU, 2022; Rinaldi et al., 2017).

Ochratoxin A (OTA) is a type of toxin produced by certain molds, specifically some species of *Aspergillus* and *Penicillium*, which are found on various agricultural products including cereals, dried fruits, wine, and coffee. The development of these fungi and subsequent ochratoxin A contamination often result from inadequate storage conditions or suboptimal agricultural practices, particularly during the drying process, where fluctuations in temperature and humidity play a significant role (Malir et al., 2016).

OTA has been identified as a significant health hazard due to its various toxic effects, especially on the kidneys. It has been recognized as a potent renal carcinogen in various animal species. Additionally, OTA is linked to weakening the immune system and interfering with biochemical processes critical for health. Recent studies also suggest that OTA may affect epigenetic factors, like DNA methylation, which can influence cell function and development, including germ cells, which are crucial for reproduction (Marin et Taranu, 2015; Zhu et al., 2017).

Perfluorooctanoic acid (PFOA) is an anthropogenic chemical known for its strong carbon-fluorine bonds, which make it highly resistant to breakdown. It has been used as stain repellent in carpeting, as a coating for packaging, and can be found in everyday items like non-stick cookware, waterproof clothing, or even in firefighting foams (Lindstrom et al, 2011). The resistance makes PFOA persistent in the environment, where it can accumulate and even increase in a concentration higher up the food chain, posing significant risks to both wildlife and humans. Consequently, widespread use has led to PFOA being detectable in various water sources, including domestic and industrial wastewater, landfill leachates, and rainwater. This contamination often results in PFOA entering soils and groundwater systems (O'Connor, 2022).

Studies in research animals have found that PFOA can cause liver damage, immune system impairment, birth defects, and delayed development in laboratory animals. Studies on workers and people living in areas with elevated levels of PFOA indicate that it may increase cholesterol levels, increase the risk of thyroid disease, decrease fertility, and affect brain function (Johansson et al., 2009). PFOA is also suspected of being an endocrine disruptor – male mice exposed to PFOA by oral gavage for 28 days have shown reduced testosterone and progesterone levels in the testes. Since PFOA can accumulate in the body for extended periods, its effects could be cumulative (Zhang et al., 2014).

1.3. Epigenetic Regulation of Germ Cell Development and Intergenerational Epigenetic Inheritance

Epigenetic mechanisms play crucial roles in establishing germ cell identity and regulating germ cell activity, which is required for precise differentiation into functional gametes and accurate transmission of genetic information to the offspring. DNA methylation and histone modifications are the most studied and best understood epigenetic mechanisms. These modifications directly affect chromatin structure, regulating the interaction between the DNA and histones, causing genes to be more or less transcriptionally active or even inactive. Also, germ cells are known to be implicated in intergenerational and transgenerational inheritance – a phenomenon in which acquired phenotypic alterations could be transmitted to the offspring (Zamudio et al., 2008).

As previously mentioned, spermatogenesis is the biological process through which spermatozoa are produced from germ cells in the testis. The formation and differentiation of germ cells are regulated via epigenetic regulation. DNA methylation plays a key role in establishing the germ cell population. This process involves the progressive transcriptional activation of a group of highly methylated and demethylated genes during germ cell epigenetic reprogramming, so-called germline responsive reprogramming (GRR) genes, and it occurs in embryonic testis. The loss of DNA methylation on GRR genes, coupled with the loss of polycomb repressor complex 1 and activation of TET1, lead to the activation of GRR genes, indicating that activation of GRR genes is a complex process regulated by

several mechanisms. Some GRR genes are known to have meiotic functions (e.g. *Brdt*, *Dazl*, *Ddx4*, *Hormad1*) (Hill et al., 2018). Therefore, the dysregulation of their epigenetic status during development, if persistent, could impact meiosis during males' adult life, which leads to a decrease in sperm production and overall fertility.

Another DNA region that could play a significant role in the development of the germ cell population is super-enhancer (SE) elements, which control gene expression essential for establishing cell identity. They consist of clusters of enhancers located upstream of coding sequences (**Figure 2.**), playing a crucial role in regulating the transcription of these genes through interactions with transcription factors (Whyte et al., 2013). SEs are known to have preserved their DNA methylation in sperm. As noted by Pott and Lieb (2015), variations in DNA methylation within CpG islands in these promoter regions could significantly affect the activity of transcription factors on these genes, thereby altering their expression. Such changes have the potential to impact the functionality of specific cell types, including neurons and germ cells. Normally, different tissues have different sets of active SE that control gene expression in those tissues.

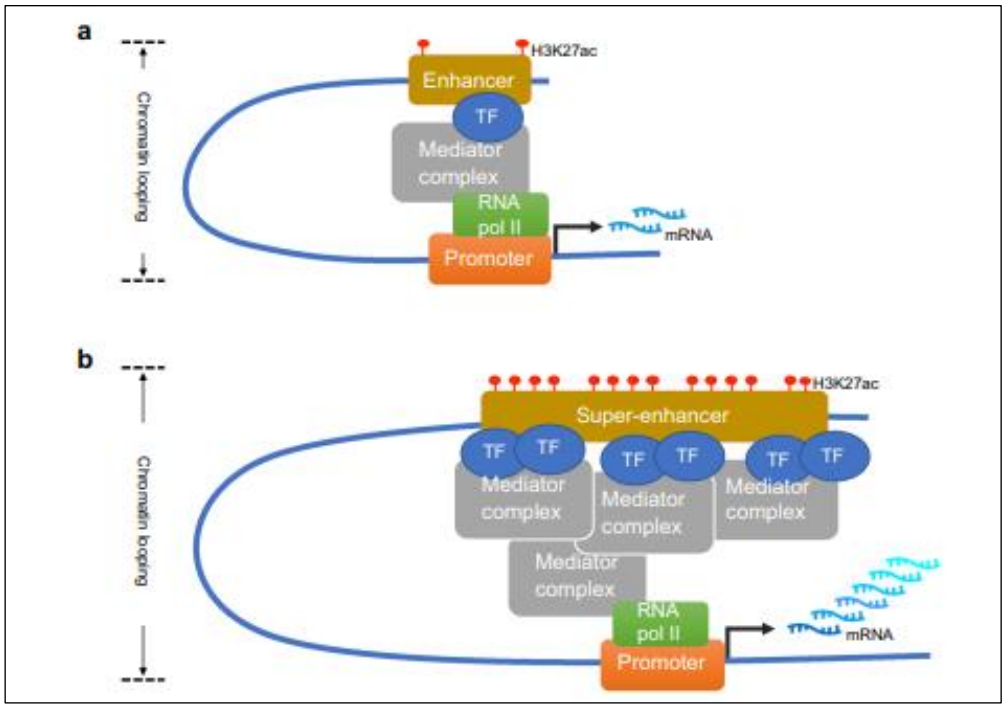

Figure 2. The structure and function of enhancer and super-enhancer complexes. (a) Schematic structure of the typical enhancer complex. (b) Schematic structure of the super-enhancer complex. H3K27ac, acetylation of histone 3 lysine 27; TF, transcription factor; RNA pol II, RNA polymerase II; mRNA, messenger RNA. Compared to typical enhancers, super-enhancers are larger, exhibit higher transcription factor and mediator density, and are frequently associated with key lineage-specific genes that control cell state and differentiation in somatic cells. Also, transcriptional output for super-enhancers is usually much higher (Chen et al., 2020).

Besides DNA methylation, histone modifications also mediate intergenerational effects. Notably, the trimethylation of sperm histone H3 at lysine 4 and 36 (H3K4me3 and H3K36me3) has been demonstrated to play a critical role in a genetic mouse model of intergenerational epigenetic inheritance (Lismer et al., 2020). Such trimethylation is important for the proper formation of double-strand breaks, which occur during meiosis in spermatogenesis. Histone H4 acetylation is crucial for post-meiotic processes essential for the formation of relaxed chromatin. While most histones are replaced by protamines during sperm development, up to 10% of histones are preserved in sperm. This retention suggests that these histones play a significant role in developmental processes.
To summarize, during germ cell development, several processes are vulnerable, such as reprograming embryonic effects, meiosis, and histone-to-protamine transition. Disruption of these processes could lead to the generation of "damaged gametes" potentially resulting in offspring with health issues.

1.4. Hormonal Regulation of Spermatogenesis

Spermatogenesis is tightly regulated by hormonal signaling, with the hypothalamic-pituitary-gonadal (HPG) axis playing a central role (**Figure 3.**). This axis coordinates the hormonal interactions necessary for the production of mature sperm capable of fertilization. The process begins with the secretion of gonadotropin-releasing hormone (GnRH) by the hypothalamus. In a pulsatile manner, GnRH stimulates the anterior pituitary gland to release two crucial hormones: follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Dwyer et Quinton, 2019; Sofikitis et al., 2008).

FSH plays a pivotal role in spermatogenesis primarily by stimulating the Sertoli cells, which are located within the seminiferous tubules and are essential for supporting and nourishing the developing spermatozoa. Sertoli cells also secrete various other factors that promote spermatocyte survival and progression. Moreover, FSH influences the phagocytic activity of Sertoli cells, helping to remove apoptotic germ cells and maintain the health of the spermatogenic environment. These cells respond to FSH by upregulating genes such as *Sox9* and *Amh* which are crucial for testicular function and germ cell development (Oduwole et al., 2018; Barrionuevo et Scherer, 2010).

On the other hand, LH targets Leydig cells in the testicular interstitial space to induce testosterone production. Upon activation, Leydig cells increase the expression of the *Star* and *Cyp17a1* genes, which are involved in steroidogenesis, thus promoting testosterone synthesis. This steroid hormone is critical for the proper development of male secondary sexual characteristics and for driving the spermatogenic process. Testosterone acts directly on the seminiferous tubules through the androgen receptor (AR), stimulating meiosis's progression and spermatozoa's maturation. Additionally, it maintains the structure and function of the male reproductive tract and influences libido and behavior (O'Hara et Smith, 2015).

The coordination of FSH and LH, along with locally produced hormones like inhibin and activin, which modulate FSH secretion through feedback mechanisms, ensures the fine-tuning of spermatogenesis.

Disruptions in this hormonal balance, such as those potentially caused by environmental pollutants, can impair spermatogenic function and lead to subfertility. These pollutants may interfere at multiple points along the HPG axis, altering hormone levels or their action at the target tissues, thereby posing significant risks to reproductive health. Understanding the mechanisms by which environmental factors influence these hormonal pathways is crucial for addressing the intergenerational consequences of such exposures (Sofikitis et al., 2008).

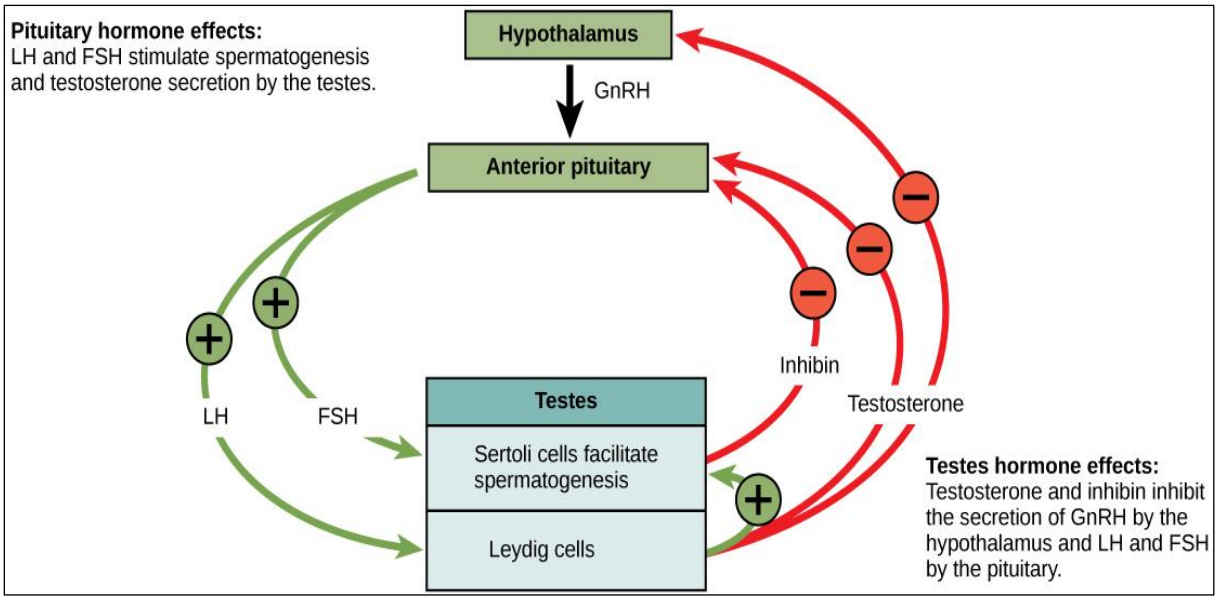

Figure 3. Diagram of the Hypothalamic-Pituitary-Gonadal (HPG) Axis: LH and FSH from the pituitary stimulate spermatogenesis and testosterone production in the testes, while testosterone and inhibin feedback to suppress GnRH, LH, and FSH secretion, maintaining hormonal balance. A negative feedback mechanism is shown. GnRH, gonadotropin-releasing hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone (Molnar et Gail, 2015).

To sum up, spermatogenesis is a complex process; it requires the coordinated actions of many hormones and transcriptional and epigenetic factors. Its complexity makes it particularly vulnerable to disruptions. Moreover, epigenetic alterations in gametes could affect not only current but subsequent generations. Studies have shown that persistent pollutants like Cd, OTA, and PFOA could induce toxicity in germ cells. However, the mechanisms underlying the epigenetic inheritance are still poorly understood.

1.5. The objectives of the study

The specific objectives of this research:

a. To assess reproductive toxicity: evaluate the effects of Cd, OTA, and PFOA on the reproductive system in male mice, focusing on parameters such as spermatozoa number, testosterone levels, and testis morphology.

b. To investigate intergenerational effects: examine whether exposure to these environmental pollutants in the parental generation (F0) affects reproductive health in subsequent generations (F1), including changes in gene expression and epigenetic modifications.

2. METHODS

2.1. Ethics Statement Using Animals

All experimental protocols obtained authorization from the French Ministry of National Education and Research. The French Ministry of Agriculture accredited the animal facility used for this study. Also, all experimental procedures respected the ethical principles specified in the Ministry of Research Guide for Care and Use of the Laboratory Animals and received approval from the local Animal Experimentation Ethics Committee (C2EA-07). Euthanasia of mice involved administering 130 mg/kg of body weight of ketamine (Virbac) and 13 mg/kg of body weight of xylazine (Elanco). Blood was collected directly from the heart, and the mice were subsequently decapitated. This euthanasia protocol complied with Annex IV of Directive 2013/118 declared by the Ministry of Agriculture, Food and Forestry of France on February 1, 2013.

2.2. Mouse Treatment and Dissection

Thirty-five-day-old outbred Swiss (RjOrl) male mice, referred to as the F0 (parental) generation, were separated into three exposure groups: Cadmium, Ochratoxin A, and Perfluorooctanoic acid. The fourth group received just water, which served as control. Exposure lasted for 35 days with doses chosen based on the tolerable daily intake (TDI) reports from ANSES and the European Food Safety Committee. The TDIs were multiplied by 500 resulting in final doses of 175 µg/day/kg for Cd, 8.50 µg/day/kg for OTA, and 750 µg/day/kg for PFOA. This multiplication factor of 500 for human dose scaling, adjusted by a correction factor of 12.3, effectively translates to a multiplication factor of approximately 40X TDI for scaling mice doses (Nair et Jacob, 2016). The substances were diluted in distilled water and delivered by gavage needle into the esophagus and stomach, with 12 males in each group, totaling 48 F0 males. After treatment, four exposed F0 males from each group were mated with unrelated and untreated females. Their progeny is referred to as the F1 generation. The remaining eight mice from each F0 group were euthanized and dissected. The F1 males were also euthanized and dissected for testis analysis at 35 days old, as well as at 60 days old for sperm analysis. The testicles and epididymides were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Epididymides were pierced, placed into Eppendorf tubes containing DMEM, and incubated at 37°C for 1 hour. The released sperm were passed through a cell strainer, pelleted, and stored at -80°C. Body weight and testis-to-body weight ratio for each mouse were also determined.

2.3. Immunofluorescence of Frozen Sections

Adult testis (F0) and 35-d-old testis (F1) were analyzed using markers such as ZBTB16 (a marker of spermatogonia) and GATA1 (a Sertoli cell marker). Right after dissection, half of the one testis from both F0 and F1 mice from each exposure group was embedded in the OCT matrix and frozen at -80°C. Testicular sections were cut using a Cryostat at a thickness of 8 µm. Samples were left to dry at room temperature for 1 hour and stored at -80°C until further use. Frozen tissue immunostaining protocol included adding 50 µL of ice-cold Fixation Buffer (4% paraformaldehyde in PBS) to each tissue section

immediately upon removal from the freezer and fixing for 8 min at 4 °C. The sections were then covered with 0.1 M glycine and incubated for 5 min at room temperature (to remove traces of paraformaldehyde), followed by three washes in PBS for 5 minutes each at 4°C. After blocking the sections in BSA containing 1X PBS-0.05% Tween (PBS-T), they were stained with primary antibodies diluted in DAKO (1:200 for ZBTB16, goat, R&D Systems; 1:100 for GATA1, rat, Santa Cruz) and incubated overnight at 4°C. The next day, slides were washed three times in PBS for 5 min at 4°C and the sections were incubated with the appropriate secondary antibody (1:500) for 1 hour at room temperature. Finally, the slides were washed three times in PBS in the same conditions as before. Sections were mounted with Vectashield Vibrant solution containing 0.001% (vol/vol) 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Images were captured using an AxioImager microscope equipped with an AxioCam MRc5 camera and operated with AxioVision software version 4.8.2 (Zeiss), using objective lenses of 20X or 40X magnification. Using ImageJ, quantitative analysis of Sertoli cells (GATA1-positive cells) and undifferentiated spermatogonia (ZBTB16-positive cells) was conducted. Positive cells were counted manually, and the number was divided by the total tubule surface area, and multiplied by 1000. The data was averaged and graphically depicted regarding the control group (±SD). Statistical significance was evaluated with a non-parametric Mann-Whitney test.

2.4. Spermatozoa Extraction and Quantification

Spermatozoa quantification was performed using epididymides collected from 2-month-old (F0 and F1) mice from each of the four groups. Briefly, the epididymides were suspended in 1 mL of 0.05% Triton buffer, carefully cut with fine scissors, and homogenized for 45 s using a PT 2500 E homogenizer (POLYTRON). The resulting homogenate was transferred to a 15 mL conical tube and stored on ice. An additional 1 mL aliquot of 0.05% Triton-X100 buffer was used to rinse the original homogenization vessel. This rinse was homogenized and combined with the primary homogenate. The rinsing and homogenizing cycle was repeated five times to obtain all spermatozoa in a final volume of 6 mL for each sample. Spermatozoa in each sample were counted using a hemocytometer. Averages were calculated from 10 squares for each biological replicate. These data were then plotted in Excel and reported as the average spermatozoa count per epididymis compared to the control group. For each exposure group, 6 to 10 biological replicates were used. Pairwise Mann-Whitney comparisons were applied to determine statistical significance.

2.5. Testosterone Quantification

Blood was drawn from the hearts of deeply anesthetized mice, collecting approximately 800 – 1,000 µL per mouse. The blood was left undisturbed at room temperature to clot for 30 min. The clot was then removed by centrifuging for 10 minutes in a refrigerated centrifuge. The supernatant (serum) obtained was stored at -80°C until further use. Testosterone levels in the serum were assayed using a commercial enzyme-linked immunosorbent assay (ELISA) based on a competitive binding with Horseradish Peroxidase (HRP) labeled testosterone antibody (Biorbyt Ltd., Cat#: orb340113, United Kingdom),

according to the manufacturer's recommendations. The results were averaged, plotted, and presented as the testosterone concentration in ng/mL.

2.6. RNA Extraction and RT-qPCR

Tissues from the control and treatment groups, stored at -80°C, were used for RNA extraction using the RNeasy Plus Mini Kit (QIAGEN). Approximately 30 mg of adult mouse testis was used for each extraction, with each group consisting of 8 biological replicates. The tissues were lysed and they were homogenized in a TissueLyser (QIAGEN) using 5-mm stainless-steel beads (69989; QIAGEN). The homogenate was subjected to DNA removal using a specialized column. Ideal binding conditions were obtained by adding a volume of 70% ethanol to the lysate. The prepared lysate was then applied to an RNeasy silica membrane. The prepared lysate was then applied to an RNeasy silica membrane. Any remaining DNA was digested by performing on-column DNase treatment using RNase-free DNase (79254; QIAGEN). The RNA was then washed using RW1 and RPE Buffers from the RNeasy Plus Mini Kit (QIAGEN) to remove potential impurities. Finally, the RNA was eluted in 50 µL of RNase-free water.

Reverse transcription was carried out using 1 µg of total RNA with iScript (1708891; Bio-Rad), following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (Bustin et al., 2009). A control reaction without reverse transcriptase (no-RT) was included for all genes tested to check for the presence of contaminating DNA, and no PCR products were detected in these controls. The housekeeping gene *Rplp37a* was selected for normalization due to its consistent expression across replicates, as verified by RNA-seq data. Primers for this study were chosen using the Primer-Blast program available at https://www.ncbi.nlm.nih.gov/tools/primer-blast/. The primers used in this experiment are listed in Annex. Statistical analysis was conducted using a non-parametric Mann-Whitney test.

2.7. DNA Extraction

DNA extraction was carried out using a DNeasy Blood & Tissue kit (69506; QIAGEN) with some modifications. Initially, spermatozoa were mechanically disrupted in AL Buffer using a TissueLyser (QIAGEN) and Tungsten Carbide Beads (69997; QIAGEN). Protein disruption was facilitated by adding dithiothreitol (DTT, 10 mM) to the lysis solution. The lysis mixture was then incubated at 56°C overnight. As per the DNA extraction protocol, the RNase A treatment step (19101; QIAGEN) was included to eliminate contaminating RNA. DNA concentration was measured using the QuantiFluor dsDNA system (E2670; Promega).

2.8. Methylated DNA Immunoprecipitation (MeDIP) and MeDIP-qPCR

The Epimark Methylated DNA Enrichment Kit (#E2600S; NEB) was employed for the DNA methylation analysis in spermatozoa. A total of 6,000 ng of DNA (spermatozoa) was sonicated using a Qsonica sonicator. The sonication settings included an efficiency of 60%, with a total sonication duration of 8 min, alternating between 20 s "on" and 20 s "off". The sonicated DNA had an average size of 300 bp and was precipitated using protein A-coated beads attached to MBD2a. This complex was then washed, and DNA was eluted using a specified elution buffer. The concentration of the DNA was quantified based on the fluorescence emitted by a dsDNA-binding dye (Promega). Approximately 7-10 ng of methylated DNA from spermatozoa was recovered after precipitation, and the non-precipitated sonicated starting material was used as "input".

For MEDIP–qPCR, both enriched DNA and input samples were diluted to equal concentrations before qPCR analysis, with equal quantities of enriched DNA and input DNA being used. The background was normalized to the *Rplp0* unmethylated region. Six biological replicates were used for the control and each exposure group. Normalized data from each gene were then compared to the corresponding controls.

2.9. Protein Extraction

Protein extraction was performed to analyze epigenetic mark occupancy using Western blotting techniques. Testicles stored at -80°C were used for this procedure. Briefly, testicles were first suspended in an ice-cold Lysis Buffer and homogenized with TissueLyser (QIAGEN) and 5-mm stainless-steel beads (69989; QIAGEN). The homogenates were then agitated for 2 hours at 4°C. Following agitation, samples were centrifuged for 20 min at 12 000 rpm at 4°C. The supernatant was carefully aspirated and transferred to a new tube kept on ice. Total protein concentrations were quantified using the Bradford assay.

2.10. Western Blot

Proteins purified through Protein Extraction were used for Western blotting. The concentrations of each protein sample, obtained from the Bradford assay, were used to calculate the exact volume containing 10 µg of protein for each sample. Western blot was performed using a rabbit 1:1000 anti-Star monoclonal antibody (D10H12, Cell Signaling Technology). Equal amounts of protein extracts (10 µg) were mixed with 10 mM Tris Buffer and Laemmli 4X Buffer, denatured at 96°C for 10 min, and run on a 4-20% gradient SDS-PAGE gel (MiniPROTEAN TGX Precast Protein Gels). Proteins were then transferred onto polyvinylidene difluoride membranes (Biorad PVDF Trans-Blot Turbo) using an electro-blotter transfer system (Trans-Blot Turbo) for 7 min. Blocking was performed using a solution of 4% milk in 1X PBS with 0,1% Tween. The primary antibody was diluted in 10 mL of this blocking solution, and the membrane was incubated with it overnight at 4°C. After three 5-minute washes with 1X PBS containing 0.1% Tween, the membrane was incubated for 1 hour in the blocking solution containing the corresponding HRP-conjugated secondary antibodies (1:10 000; GE Healthcare).

Following another series of three 5-minute washes with 1X PBS containing 0.1% Tween, the membrane was coated with Amersham ECL Prime Western Blotting Detection Reagent (RPN2232; Amersham) for detection. Specific protein expression corresponding to the antibody was then detected and measured using a molecular imager (ChemiDoc XRS + System with Image Lab Software). Ponceau Red-stained bands were used to evaluate protein loading and normalize the levels of the belonging protein to the total amount of proteins for each sample. The intensity of these bands was measured using Fiji: ImageJ software.

2.11. Statistical Analysis

For each specific experimental procedure, the minimum number of animals was used, according to the requirements of the EU Ethics Committee. A non-parametric Mann-Whitney test was used to assess the statistical significance of the assays conducted in this research.

3. RESULTS

3.1. The Effects on Body and Organ Weights, Spermatozoa Count and Testosterone Levels

3.1.1. Body Weight

To determine the general toxicity of Cadmium, Ochratoxin A, and Perfluorooctanoic acid in male mice, we measured the body weights of two-month-old mice in each exposure group and compared them to the control. As shown in **Figure 4.**, the F0 mice treated with OTA and PFOA did not show a significant change in body weight, yet there is a ~6% increase for the Cd-treated mice. Also, no significant changes in the testis-to-body weight ratio for these mice were detected.

For the F1 progeny mice body weights, parental exposure shows a ~9% decrease in the Cd group, a ~6% increase in the OTA group, and ~8% in the PFOA group. The testis-to-body weight ratio increased significantly for the Cd group by ~18%. For OTA and PFOA, that ratio tends to increase (**Figure 5.**).

Previous studies by the team showed that exposure to each of these three toxicants negatively affected organs-to-bodyweight ratios, including organs like the liver, kidney, prostate, and epididymis (oral communication, dr. sc. Fatima Smagulova), suggesting general systemic toxicity induced by all 3 toxicants.

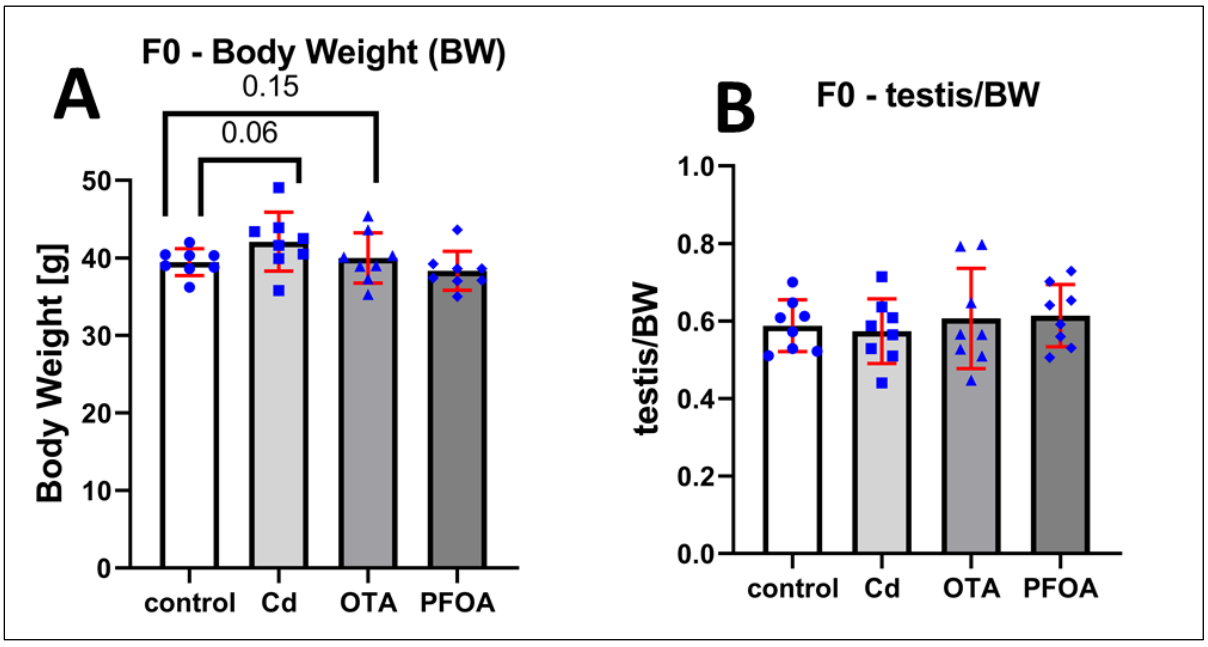

Figure 4. Body weights (A) and testis-to-bodyweight ratios (B) for F0 generation. Data are represented as mean ± SD. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$, non-parametric Mann-Whitney test.

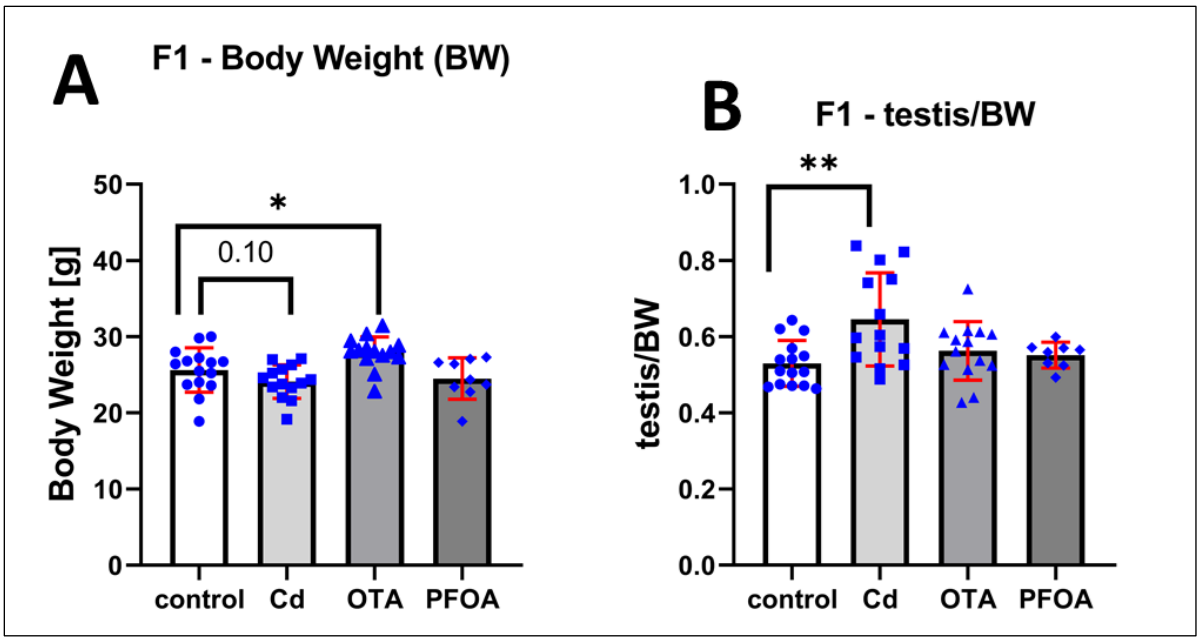

Figure 5. Body weights (A) and testis-to-bodyweight ratios (B) for F1 generation. Data are represented as mean ± SD. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$, non-parametric Mann-Whitney test.

3.1.2. Spermatozoa Count

To assess the further effects of our 3 toxicants – on the male reproductive system specifically – we performed a quantitative analysis of spermatozoa per epididymis for each exposure group and compared them to the control group. In 2-month-old mice from the F0 generation, there is a significant reduction of sperm count in all 3 exposure groups: a reduction of ~42% in the Cd-treated group, ~38% in the OTA group, and ~30% in the PFOA group. Similarly, there is a significant sperm reduction in the F1 generation in 2-month-old mice in the Cd group (~33%) and the OTA group (~32%). For the PFOA group, there is a tendency to decrease in spermatozoa numbers (by ~15%). (**Figure 6.**) These data are a strong indicator of impaired spermatogenesis, as spermatozoa count is an essential parameter of reproductive health.

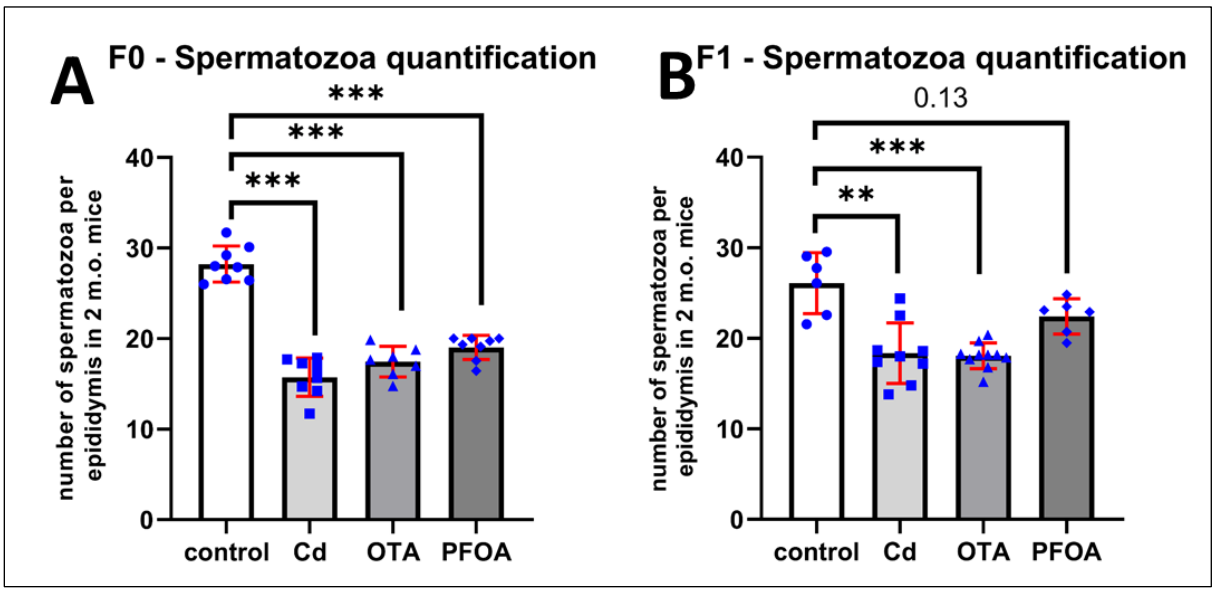

Figure 6. Spermatozoa quantification in 2-month-old mice. (A) Results for F0 generation (effect of direct exposure). (B) Results for F1 generation (effect of parental exposure). Data are represented as mean ± SD. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$, non-parametric Mann-Whitney test.

3.1.3. Testosterone Levels

Testosterone (T) is an essential hormone for male gametogenesis. Thus, we decided to measure the level of T in the blood serum. To this end, we used the blood serum of males taken during mice dissection. The decreased testosterone levels (**Figure 7.**) observed in exposed mice and their progeny (F0 and F1 2-month-old mice) highlight endocrine disruption as a significant consequence of toxicant exposure. Our ELISA analysis revealed a substantial reduction in testosterone levels in F0 across all treatment groups compared to the control: ~46% in the Cd-treated group, ~50% in the OTA-treated group, and ~30% in the PFOA-treated group. As for F1, we detected a significant reduction in OTA and PFOA groups (~63% and ~82%). The Cd group also tended to decrease testosterone levels, though it was not statistically significant (~66%).

In summary, there is an effect of all tested compounds on spermatozoa decrease, suggesting that continuous exposure to these pollutants had a strong impact on reproductive health. The decrease in testosterone levels might contribute to the spermatozoa decline, as testosterone is essential for male gamete production. Studies have shown that androgen receptor-deficient mice are infertile, highlighting the role of testosterone.

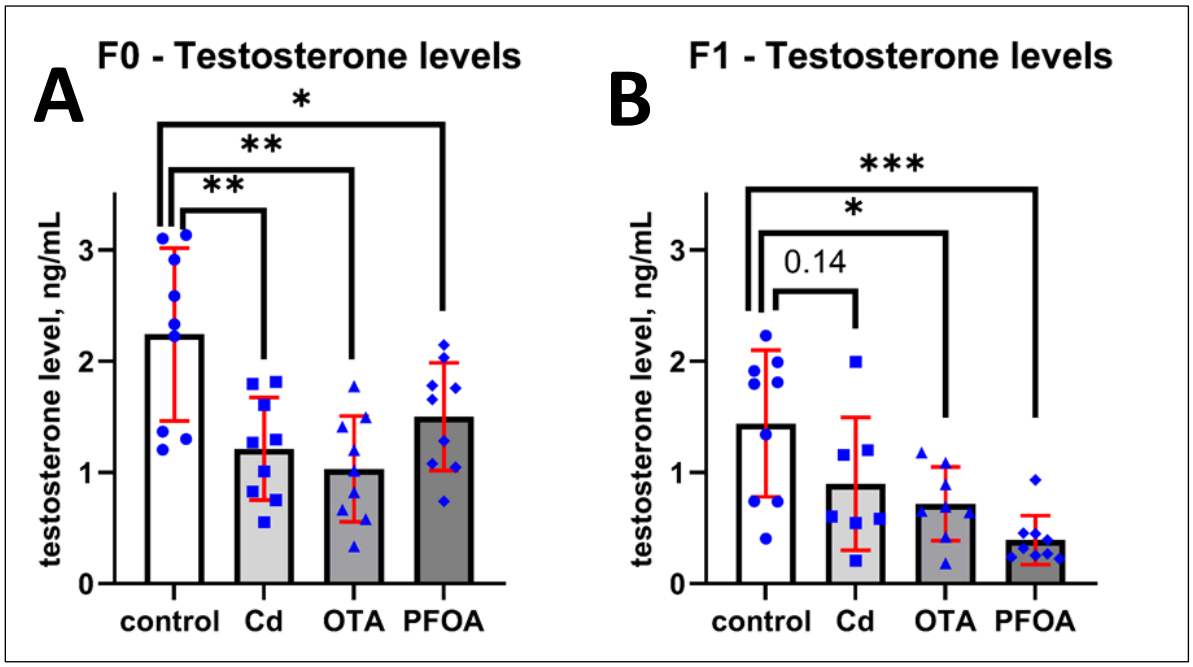

Figure 7. Testosterone levels in blood serum. (A) Results for F0 generation (effect of direct exposure). (B) Results for F1 generation (effect of parental exposure). Data are represented as mean ± SD. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$, non-parametric Mann-Whitney test.

3.2. Immunostaining Analysis of Testis

To investigate potential changes in the number of germ and Sertoli cells, we performed immunostaining on testicular tissue using antibodies specific for undifferentiated spermatogonial stem cells (ZBTB16-positive cells) and Sertoli cells (GATA1-positive cells). To proceed with our hypothesis, we prepared frozen testis sections and immunostained them against these markers. The quantitative analysis of ZBTB16-positive or GATA1-positive cells per tubule showed no significant alterations in F0 generation (*Annex*). However, as shown in **Figure 8.**, we observed a significant increase in GATA1-positive cells in the Cd and PFOA groups (~60 and ~30%) in the F1 generation, suggesting that parental exposure to these toxicants affected the somatic cell numbers in the progeny. The OTA group exhibited only a tendency toward an increase. Additionally, across all three toxicant groups, there was no significant change in ZBTB16-positive cells, with the Cd group showing only a tendency to increase. This suggests that parental exposure to these three toxicants did not significantly alter the number of undifferentiated spermatogonia in the F1 generation.

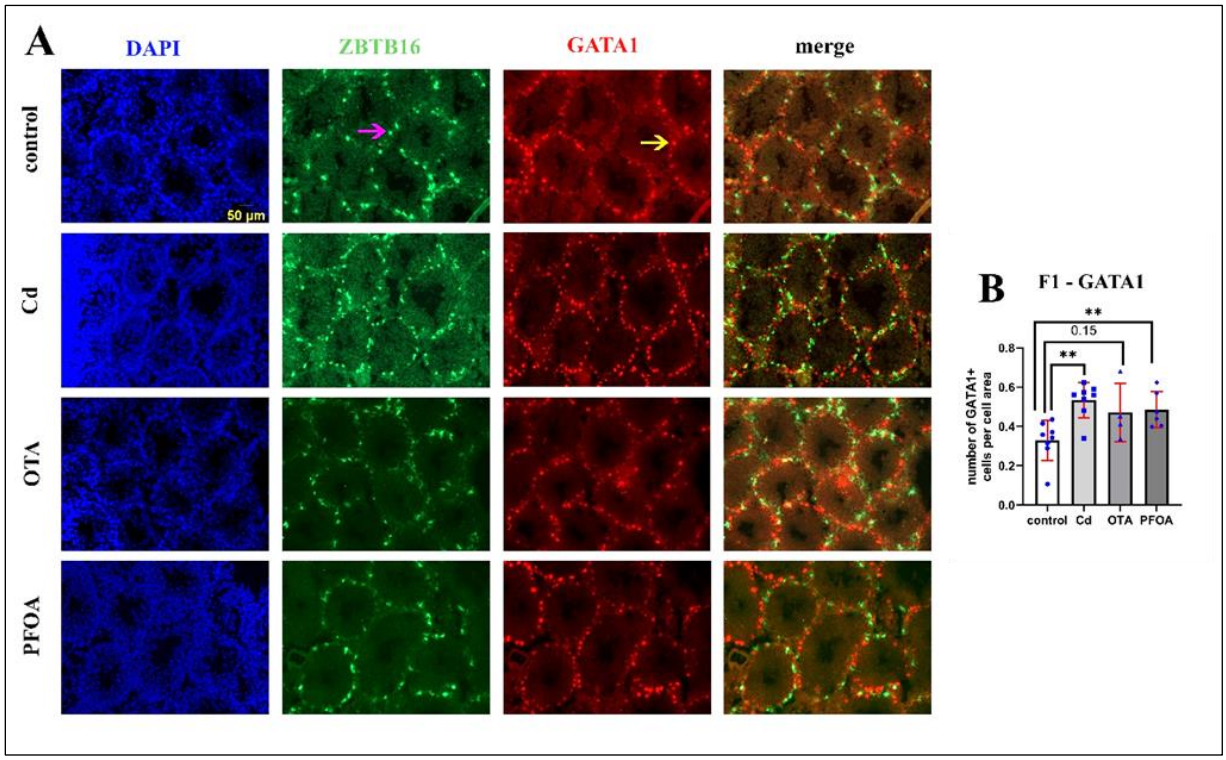

Figure 8. (A) A representative image of testis sections from 35-day-old F1 generation mice, immunostained with ZBTB16 (green, a marker of spermatogonia; positive cells indicated by pink arrow) and GATA1 (red, a marker of Sertoli cells; positive cells indicated by yellow arrow), is shown. Images from the control group and all 3 toxicant groups were captured using 20X objectives. (B) Quantitative analysis of GATA1-positive cells across the 3 toxicant groups compared to the control group is presented. Data are represented as mean ± SD. ** $p < 0.01$, non-parametric Mann-Whitney test.

3.3. mRNA Levels of Cell Type-Specific Markers, GRR Genes and Hormonal Signaling Genes

Note: RT-qPCR results for cell type-specific genes and GRR genes are provided in the *Annex*.

3.3.1. Cell type-specific markers

In order to reveal if continuous paternal exposure alters the testis cell populations, we conducted RT-qPCR analysis using cell type-specific markers to evaluate cell type-specific changes further. We designed primers and performed the analysis for markers specific to spermatogonia (SG), spermatocytes (SC), spermatids (ST), and Sertoli cells. Single-cell sequencing data from Green et al. (2018) served as the source for these specific markers. Expression for each gene in each exposure group was compared to the control. No significant changes were observed in F0 mice from all 3 exposure groups. We can only determine significant changes in expression for two spermatocyte-specific genes (*Hormad1* increase in the OTA-treated group and *Piwil1* reduction in the PFOA-treated group). As for F1, the most notable results are the ones for spermatogonia-specific genes (*Kit* and *Stra8*) – there is a significant decrease in those gene expressions for the groups treated with Cadmium and Ochratoxin A and an increase for the Perfluorooctanoic acid-treated group. Combined with the absence of significant changes in ZBTB16-positive cells (*Immunostaining Analysis of Testis*), these data suggest that changes in F1 generation do not derive from cell population changes, but rather from the perturbation in their regulation, possibly via epigenetic mechanisms. (*Annex*)

3.3.2. Germline responsive reprogramming genes and genes regulated via super-enhancer elements

Next, we determined the mRNA levels of germline responsive reprogramming (GRR) genes, as well as for super-enhancer (SE) elements. GRR genes are necessary for the germ cell population establishment, and SE elements control them. For the groups treated with Cd and OTA in the F0 generation, there is no global change in gene expression compared to the control. The group treated with PFOA showed an overall reduction in gene expression, with significance for some of them (*Sox9*, *Spo11*, *Tdrd1*, *Dmrtb1*). Similarly, gene expression for the Cd group in the F1 generation remained unaltered. However, a considerable number of genes in the OTA group increased in gene expression, with the most drastic changes in *Dazl*, *Stk31*, *Spo11*, and *Dmrtb1*. Finally, gene expression significantly increased for most of the genes in the PFOA group, with the most dramatic changes in the same genes as in the OTA group - *Dazl*, *Stk31*, and *Dmrtb1*.

Our results showed that F0 generation males exhibited minimal changes in GRR gene expression, however, significant impacts were observed in males from the F1 OTA and PFOA groups. These findings suggest that alterations in GRR and SE gene expression observed in the F1 generation may have been inherited through sperm from the F0 males.

3.3.3. Hormone signaling genes

Since testosterone is critical for regulating spermatogenesis and overall male reproductive health, the observed reduction in its levels (**Fig. 7.**) led us to investigate whether there is a change in the expression of the genes involved in the hormonal signaling pathway. Similar to the analysis of the previous group of genes in F0 and F1 generations, we did not detect strong changes in gene expression for the Cd group (*Annex*). In the OTA and PFOA groups, we determined a global decrease in gene expression in the F1 generation – this finding correlates with the reduction in spermatozoa (**Fig. 6.**). The OTA group showed a general decrease for both generations, with *Star* reduction being the most prominent one, correlating with the reduction of testosterone levels (**Figure 9.**)

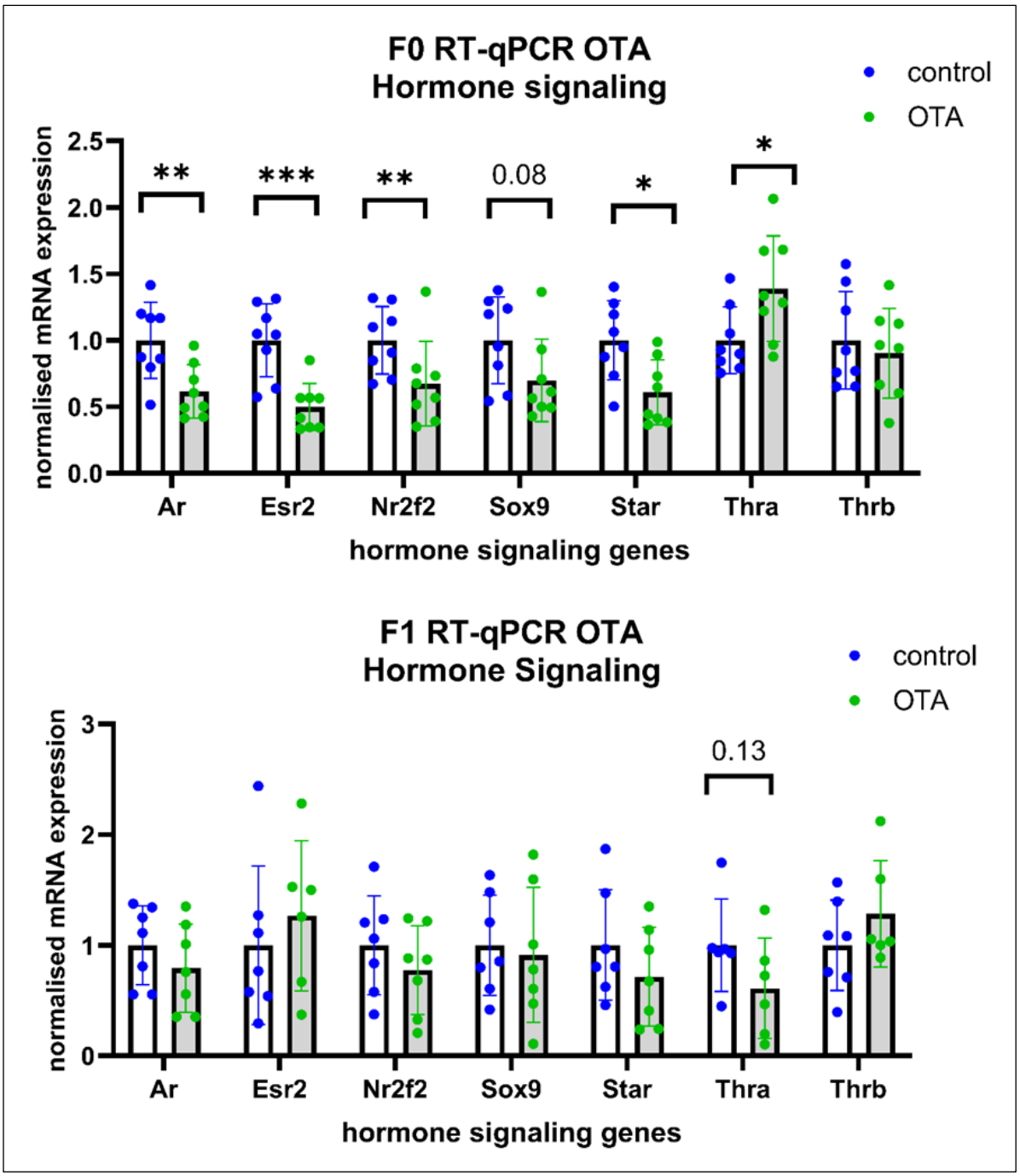

Figure 9. Quantitative RT-qPCR analysis of hormonal signaling genes – OTA group, in 2-month-old testis from the F0 and 35-day-old testis from the F1 generation. Gene expression was normalized to the housekeeping gene *Rplp37a*. Data are represented as mean ± SD. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$, non-parametric Mann-Whitney test.

Following the previous results, PFOA again showed the most remarkable variations in gene expression, especially for the F0 generation, where significant *Star* reduction (~40%) is also present. However, an increase in *Star* expression (1.7 times) was observed in the F1 generation (**Figure 10.**)

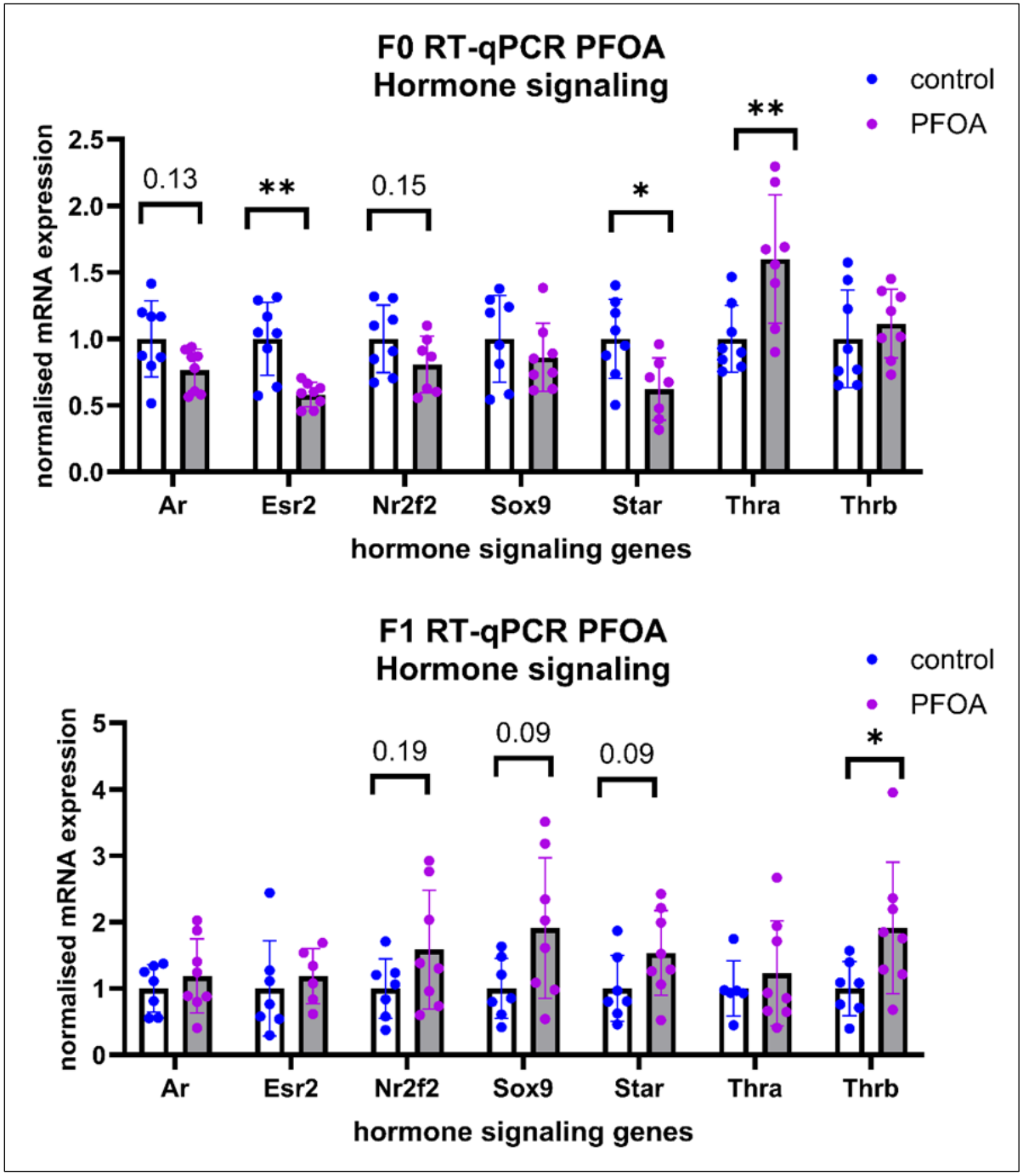

Figure 10. RTqPCR results for the hormonal signaling genes – PFOA group, in 2-month-old testis from the F0 and 35-day-old testis from the F1 generation. Gene expression was normalized to the housekeeping gene *Rplp37a*. Data are represented as mean ± SD. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$, non-parametric Mann-Whitney test.

3.4. Effect of PFOA Treatment on the Level of Star Protein

To investigate the impact of direct exposure to PFOA on Star protein expression, we performed Western Blot analysis on F0 testis samples from two groups of mice: those treated with PFOA (n=5) and control mice (n=5). The normalized Star protein levels in the PFOA-treated group were reduced by approximately 75% compared to the control group ($p < 0.05$), indicating a statistically significant decrease in Star expression following PFOA treatment (**Figure 11.**), which is consistent with the RT-qPCR data. The band intensity was normalized to the Ponceau red stain.

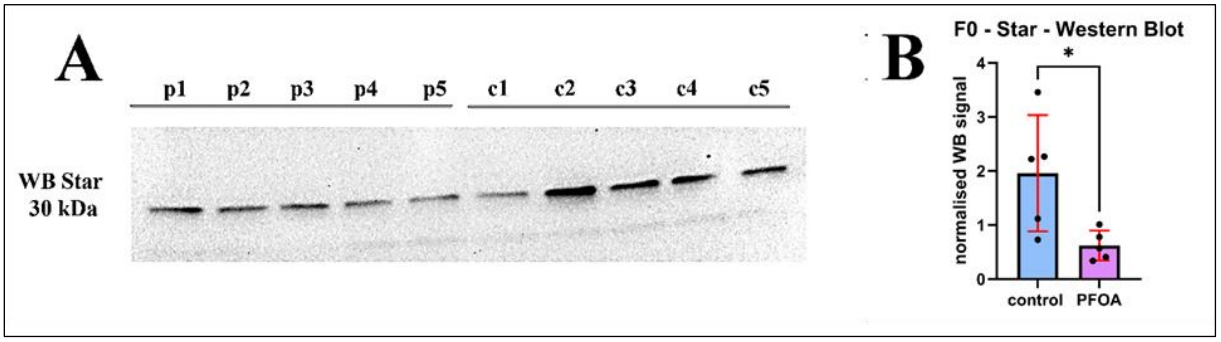

Figure 11. Western Blot analysis of Star protein expression in testis samples from control and PFOA-treated mice. (A) Representative Western Blot image showing normalized Star reduction in PFOA-treated mice compared to the control mice, p1-p5 are PFOA-derived samples, and c1-c5 are control samples. (B) Quantification of Star protein levels. Data are presented as mean ± SD (n=5 per group). *$p < 0.05$ nonparametric Mann-Whitney test.

3.5. Epigenetic Effects Promoted by Environmental Pollutants, and Intergenerational Effects on Spermatogenesis

As germ cell formation and differentiation are epigenetically regulated, and because spermatozoa (and oocytes) are the only cells transmitted to future generations, we tested whether exposure to environmental pollutants altered DNA methylation in the spermatozoa of mice. That is why MeDIP-qPCR was performed, as described in the Materials and Methods section. GRR genes and SE elements were used as gene markers in this study due to their demonstrated importance for germ cell development in previous research. We examined the changes in these markers in F0 generation. Also, we wanted to investigate how alterations in these epigenetic marks influence gene expression by integrating these epigenetic marks with gene expression data. The goal was to determine the intergenerational effect of how direct exposure to environmental pollutants affects gene expression in the F1 generation. That is why MeDIP-qPCR from the F0 generation was compared to the RT-qPCR results from the F1 generation for the same gene markers. Groups treated with PFOA and OTA (F0) and their progeny (F1) were used for this purpose.

Most GRRs and SEs are localized in the promoter region meaning that their methylation should reversely correlate to the gene expression. Results depicted in **Figure 12.** and **Figure 13.** show that correlation indeed occurs for all of the GRR genes chosen, suggesting that direct exposure to OTA and PFOA does change the gene expression in the offspring, which is caused by an epigenetic alteration in the paternal spermatozoa.

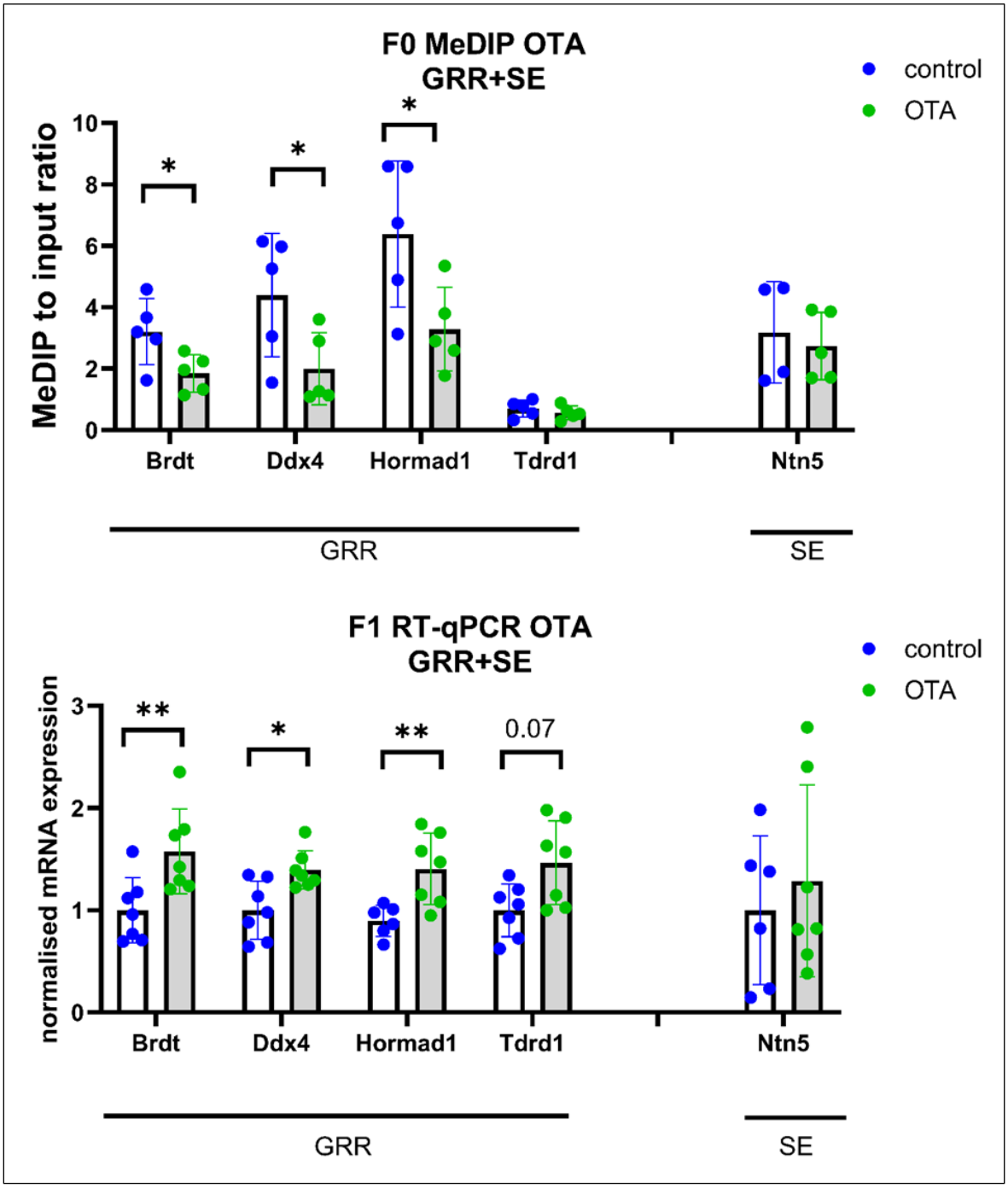

Figure 12. MeDIP results for the GRR+SE – OTA group, in 2-m-old mice sperm from the F0 generation, and the RT-qPCR results for the same gene markers – OTA group, in 35-d-old mice testis from the F1 generation. Data are represented as mean ± SD. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$, non-parametric Mann-Whitney test.

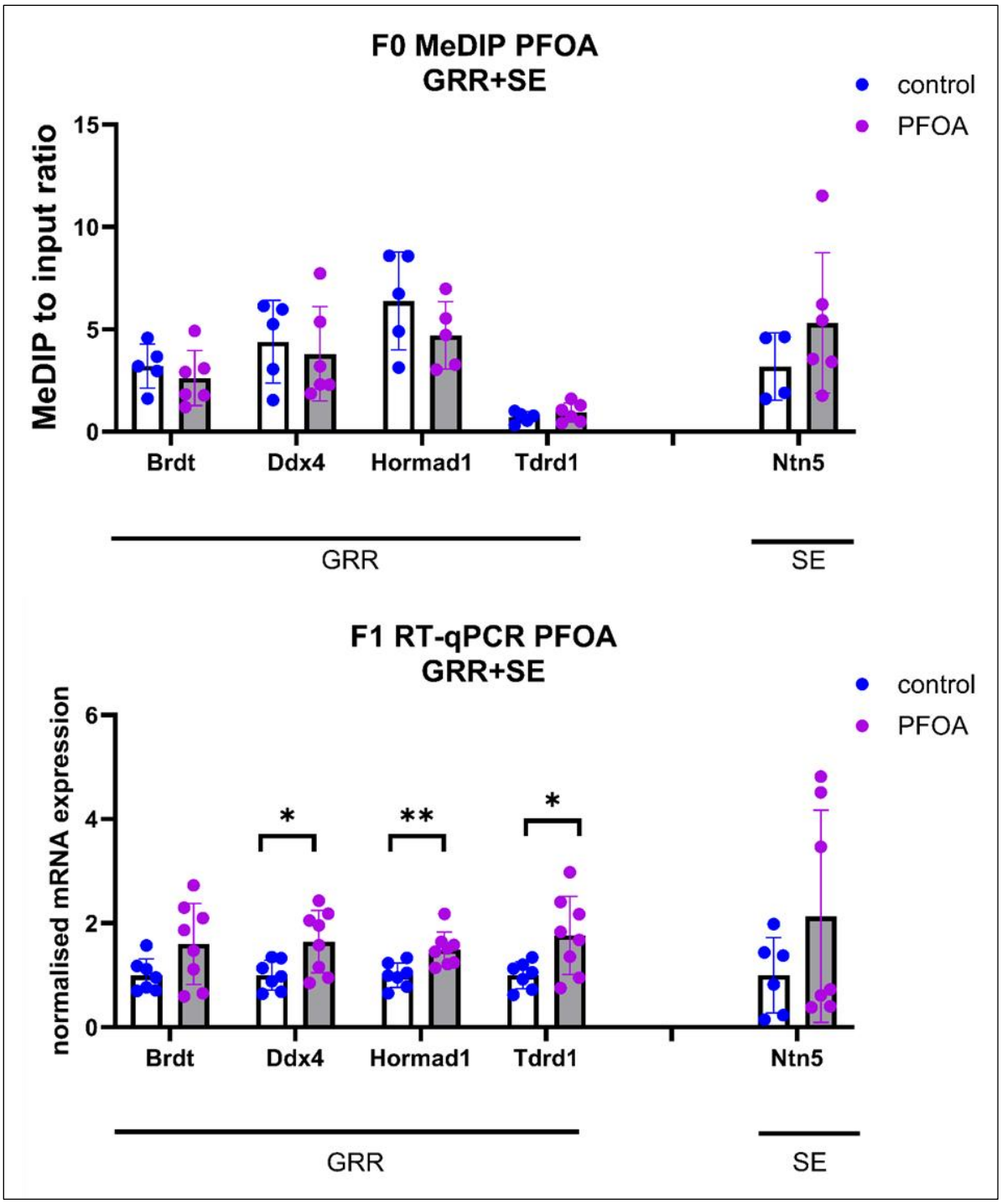

Figure 13. MeDIP results for the GRR+SE – PFOA group, in 2-m-old mice sperm from the F0 generation, and the RT-qPCR results for the same gene markers – PFOA group, in 35-d-old mice testis from the F1 generation. Data are represented as mean ± SD. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$, non-parametric Mann-Whitney test.

Our analysis revealed that the promoters of *Brdt*, *Ddx4*, *Hormad1*, and *Tdrd1* were hypermethylated in the F0 generation, which could be the reason for the decreased gene expression of these genes in F1.

4. DISCUSSION

Environmental pollutants are pervasive in our modern world, with far-reaching impacts on human and wildlife health. Substances like Cadmium (Cd), Ochratoxin A (OTA), and Perfluorooctanoic acid (PFOA) are particularly concerning due to their persistence in the environment and their ability to disrupt biological processes. Recognizing the urgent need to address these risks, the HBM4EU (Human Biomonitoring for Europe) initiative has prioritized these substances for comprehensive risk assessment. These toxicants can infiltrate various aspects of daily life, from the food we eat to the water we drink, leading to a range of health issues including reproductive toxicity, hormonal imbalances, and genetic instability.

The results demonstrate that exposure to Cadmium, Ochratoxin A, and Perfluorooctanoic acid induces significant general toxicity in mice, affecting body weight, testis-to-body weight ratio, sperm count, and testosterone levels. Even though the chosen doses did not significantly affect body weight and testis-to-body weights, they were sufficient to cause a substantial decrease in spermatozoa count across all 3 exposure groups, which is a strong indicator of impaired spermatogenesis. This finding is consistent with known mechanisms of toxicity for these compounds, such as oxidative stress and disruption of cellular processes essential for sperm production. The reduced sperm count is particularly concerning as it directly correlates with fertility potential. Therefore, we quantified testosterone levels. The observed decrease in testosterone in exposed mice highlights endocrine disruption as a significant consequence of toxicant exposure. Testosterone is critical for the regulation of spermatogenesis and overall male reproductive health. The reduction in testosterone suggests that these toxicants interfere with the hormonal regulation of reproductive functions, which could lead to decreased fertility and adverse effects on offspring health.

We observed a significant decrease in testosterone in F1, even in the absence of exposure. Although we did not determine changes in DNA methylation of hormone signaling genes (data not shown), we suggest that the alterations in their expression may be controlled by other mechanisms, such as histone modifications.

Previous studies have already indicated that these pollutants can influence fertility through a decrease in sperm quantity (Kumar and Sharma, 2019; Zhang et al., 2017; Bach et al., 2016), however precision at the testicular level is yet to be established. The immunostaining results for GATA1 and ZBTB16 antibodies provide critical insights into the impact of Cd, OTA, and PFOA on the cellular composition of the testes across generations. The lack of significant change in the number of GATA1-positive cells or ZBTB16-positive cells for the F0 generation suggests that the initial exposure to our 3 pollutants does not immediately affect the populations of these critical cell types within the testes. Despite the observed general toxicity, the stability of these cell populations in the F0 generation suggests that the immediate cellular environment within the testes may initially resist structural alterations induced by these

toxicants. In the F1 generation, however, a significant rise in Sertoli cell numbers could indicate a compensatory response to the toxicant-induced damage observed in the F0 generation. Since Sertoli cells are essential for nurturing developing sperm cells and maintaining the integrity of the blood-testis barrier, their increase could be an attempt to mitigate the detrimental effects of toxicant exposure observed in the earlier generation. The increase in Sertoli cells might also be interpreted as a stress response, potentially indicating ongoing damage or an attempt to maintain spermatogenic efficiency under toxicant pressure. However, this compensatory mechanism appears insufficient to prevent the observed reproductive toxicity. For the ZBTB16-positive undifferentiated spermatogonia, their stability suggests that the early stages of spermatogonial development are less susceptible to alterations from these toxicants, or it could imply that the effects of Cd, OTA, and PFOA are more pronounced at later stages of spermatogenesis, possibly through disruption of the hormonal milieu or direct damage to more mature sperm cells.

Examining germline responsive reprogramming genes and super-enhancers is crucial, as these genes play pivotal roles in the regulation of spermatogenesis and the maintenance of genetic stability. In the F0 generation, no significant changes were detected in the expression of these genes in the Cd and OTA groups, suggesting that immediate toxicant exposure does not profoundly disrupt these pathways. However, the PFOA-treated group exhibited a notable reduction in gene expression, indicating a potential impairment in the regulation of spermatogenesis. In the F1 generation, the effects of parental exposure were more pronounced in the OTA and PFOA groups. The OTA group showed increased expression of several genes, notably *Dazl*, *Stk31*, *Spo11*, and *Dmrtb1* which suggests an adaptive or stress response aiming to enhance germline stability and spermatogenesis. Similarly, the PFOA group demonstrated significant upregulation of these genes, indicating a robust response to counteract the negative effects observed in the F0 generation. These findings align with the observed increase in GATA1-positive Sertoli cells, suggesting a coordinated response to maintain spermatogenic efficiency and protect germline integrity.

Investigating hormone-signaling genes is critical as they regulate various aspects of reproductive function, including spermatogenesis and steroidogenesis. In our study, we did not observe significant changes in hormone-signaling gene expression in the Cadmium-exposed F0 generation. On the other hand, a general decrease in these genes was noted in the F1 generation. This reduction aligns with the decreased spermatozoa count observed in the F1 Cadmium group, suggesting that Cadmium exposure may disrupt hormonal regulation of spermatogenesis in a way that manifests more clearly in the subsequent generation. This disruption might occur through mechanisms affecting testosterone biosynthesis enzymes or other hormonal pathways, leading to impaired reproductive function in the offspring.

The OTA group displayed a general decrease in hormone signaling gene expression across both generations, with the most pronounced reduction in Star, a key protein in steroid hormone biosynthesis.

This persistent downregulation suggests a long-term disruption of hormonal balance, potentially contributing to the observed reproductive toxicity.

Notably, out of three compounds, PFOA exposure revealed the most dramatic changes. The significant reduction in hormone signaling genes, including Star, in the F0 generation, indicates severe disruption of hormonal pathways. Interestingly, the F1 generation showed an increase in Star expression, suggesting a potential compensatory mechanism to restore hormonal balance and support spermatogenesis. This increase aligns with the upregulation of germline responsive genes, highlighting a coordinated adaptive response to PFOA-induced stress.

To further confirm the detrimental effect of direct PFOA exposure on the hormone-signaling pathway, Western blot analysis was performed on F0 mice testis samples to quantify Star protein levels. Star (Steroidogenic Acute Regulatory Protein) plays a crucial in transporting cholesterol into mitochondria, which is a key step in steroid hormone biosynthesis. The observed decrease in Star protein levels aligns with earlier mRNA findings, indicating impaired testosterone synthesis in PFOA-exposed mice. This disruption in hormone production corresponds with previously observed reductions in testosterone levels, potentially contributing to decreased sperm count and adverse effects on spermatogenesis.

Finally, the investigation into the epigenetic modifications induced by environmental pollutants, OTA and PFOA specifically, in the directly exposed F0 generation and their subsequent impact on the F1 generation provides a crucial layer of understanding regarding the long-term reproductive toxicity of these pollutants. As mentioned, GRR genes play essential roles in establishing the germ cell population. The activation of those genes is controlled by various mechanisms that are regulated by complex network. Therefore, it is likely that disruptions in their epigenetic status during spermatogenesis could affect meiosis in adult males, as well as in their progeny. Variations in mRNA levels of these genes are often correlated with changes in the DNA methylation level of these genes. That is why we investigated the state of DNA methylation in these regions using MeDIP-qPCR and sperm DNA. By employing MeDIP-qPCR for GRR genes and super-enhancers (SE) in the F0 generation, and comparing these results with RT-qPCR data from the F1 generation, we aimed to elucidate the intergenerational transmission of epigenetic changes.

MeDIP-qPCR, which measures DNA methylation levels, should exhibit an inverse correlation with gene expression levels measured by RT-qPCR. Increased DNA methylation typically leads to gene silencing by blocking transcription factor binding or recruiting repressive chromatin remodeling complexes. Indeed, for both the OTA and PFOA groups, DNA methylation in the F0 generation translated into global changes in the F1 generation's gene expression profiles. This pattern suggests that epigenetic reprogramming induced by these compounds leads to compensatory upregulation of critical spermatogenesis genes, potentially as a mechanism to counteract toxicant-induced damage.

5. CONCLUSIONS

After carrying out this experimental work, we can conclude:

- **Reproductive Toxicity of Environmental Pollutants**:
  Exposure to Cadmium (Cd), Ochratoxin A (OTA), and Perfluorooctanoic acid (PFOA) has significant negative impacts on reproductive health in mice, with PFOA having the most profound effect. These pollutants reduce sperm count, disrupt hormone levels, and affect the overall reproductive system, indicating potential risks for human fertility as well.

- **Intergenerational Effects**:
  The toxic effects of these pollutants impact not only the directly exposed generation (F0) but also their offspring (F1). Compensatory changes in gene expression in the F1 generation suggest an adaptive response to parental exposure, which highlights the importance of considering long-term and generational impacts in environmental health assessments.

- **Epigenetic Modifications**:
  Epigenetic changes, such as DNA methylation, play a crucial role in mediating the toxic effects of OTA and PFOA. The inverse correlation between DNA methylation and gene expression underscores the complex relationship between environmental exposures and epigenetic regulation.

- **Compensatory Mechanisms**:
  The observed compensatory upregulation of key genes involved in spermatogenesis and hormone signaling in the F1 generation suggests an attempt by the body to counteract the detrimental effects of these pollutants. However, the long-term efficacy and potential side effects of these compensatory mechanisms require further investigation.

These findings highlight the importance of strict regulations to limit exposure to harmful environmental pollutants. By understanding how these substances affect reproductive health, we can develop better strategies to protect human health and reduce the risks associated with environmental pollutants.

Further research should aim to clarify the exact molecular pathways affected by these pollutants and their potential impacts on reproductive health across multiple generations. Additionally, it is important to examine the effects of these toxicants on ovaries to fully understand their impact on female reproductive health. This will help us better understand the wider effects of environmental toxicants and guide public health policies to reduce exposure and protect reproductive health.

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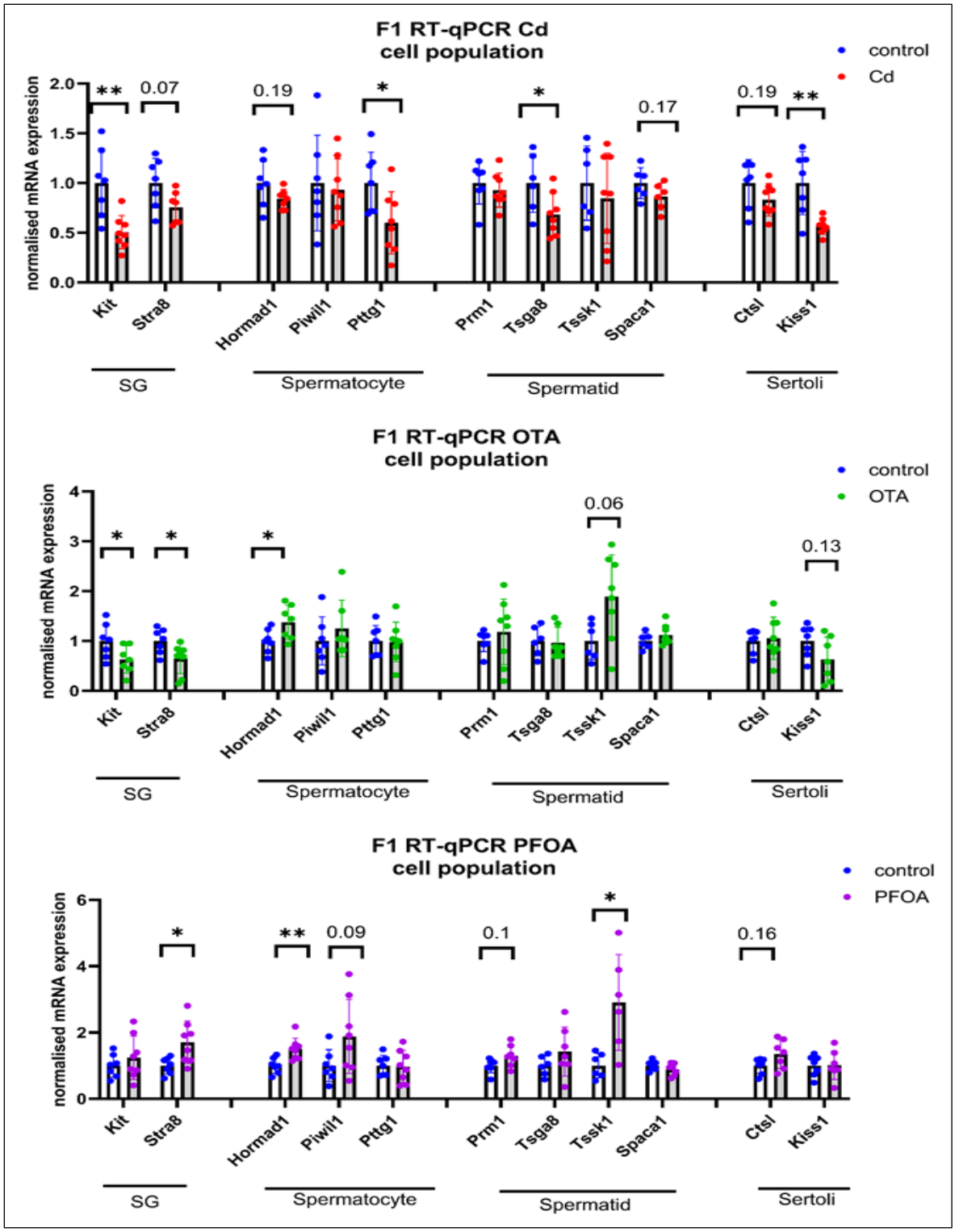
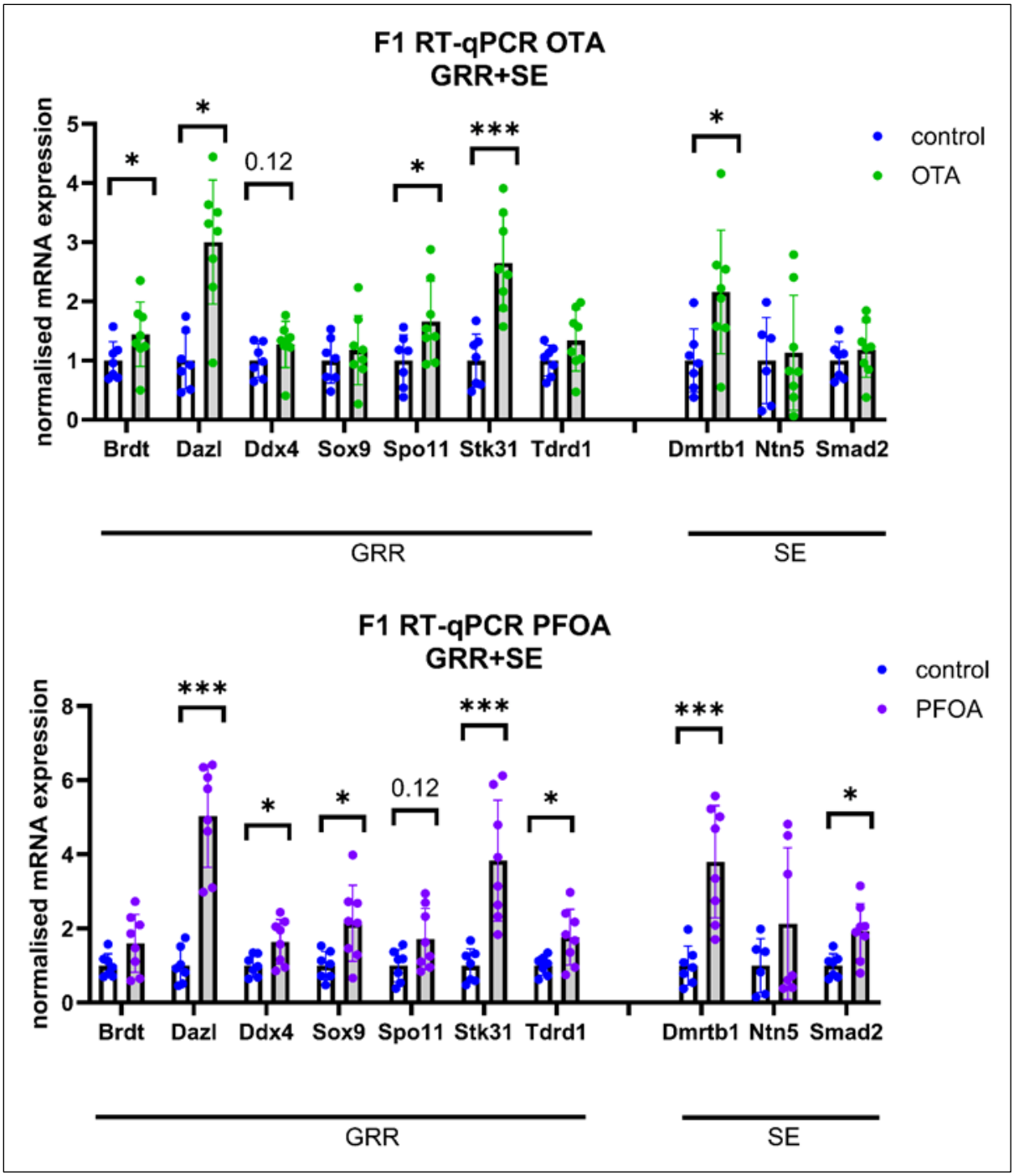
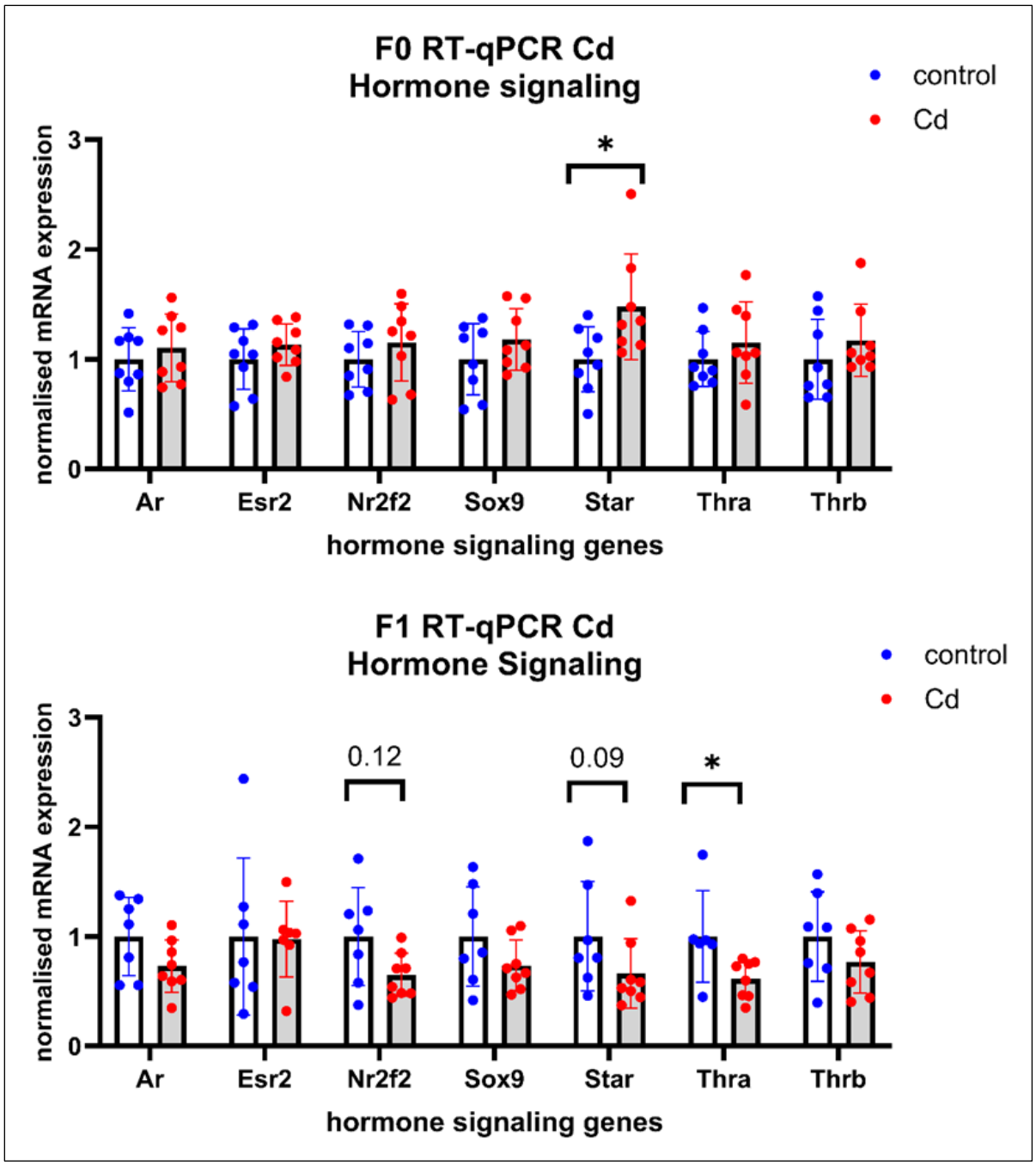
ANNEX



Supplementary Figure 1. Quantitative RT-qPCR analysis of cell population genes for all 3 toxicant groups, in 35-d-old testis from the F1 generation. Gene expression was normalized to the housekeeping gene *Rplp37a*. Data are represented as mean ± SD. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$, non-parametric Mann-Whitney test.



Supplementary Figure 2. Quantitative RT-qPCR analysis of GRR+SE markers for OTA and PFOA groups, in 35-d-old testis from the F1 generation. Gene expression was normalized to the housekeeping gene *Rplp37a*. Data are represented as mean ± SD. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$, non-parametric Mann-Whitney test.



Supplementary Figure 3. Quantitative RT-qPCR analysis of hormonal signaling genes – Cd group, in 2-month-old testis from the F0 and 35-day-old testis from the F1 generation. Gene expression was normalized to the housekeeping gene *Rplp37a*. Data are represented as mean ± SD. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$, non-parametric Mann-Whitney test.

Supplementary Table 1. List of primers for RT-qPCR and MeDIP used in this work

ID	Gene Name	Biological Process	Forward Primer	Reverse Primer
Ar	androgen receptor(Ar)	negative regulation of transcription from RNA polymerase II promoter	GTCCTTCACTAATGTCAACTCCA	CCACTGGAATAATGCTGAAGAG
Brdt	bromodomain, testis-specific(Brdt)	chromatin organization, male meiosis	GAGGGTTGTGCTGAAGGCCC	TGATGGTGTAATAGTCAGGCAGC
Ctsl	cathepsin L(Ctsl)	proteolysis, thyroid hormone generation	GCTAGCCGCCTCAGGTGTTT	TTCCACTGGTGCCACTCTGC
Dazl	deleted in azoospermia-like(Dazl)	oocyte maturation, regulation of translation, germ cell development	CAGTATGTTCAGGCATATCCTC	ATTCATTGGGCAAAATATCAGC
Ddx4	DEAD box helicase 4(Ddx4)	male meiosis	ACAGGATGTCCCCGCATGGC	TCCCATGACTCGTCATCAACTGGA
Dmrtb1	DMRT-like family B with proline-rich C-terminal, 1(Dmrtb1)	regulation of transcription	GCTACCCGGATCAGCACCTG	CTCAGAGGATGCCCGTAGTCG
Esr2	estrogen receptor 2 (beta)(Esr2)	negative regulation of transcription from RNA polymerase II promoter, ovarian follicle development	GTCAGGCACATCAGTAACAAGG	TGAGCATTCAGCATCTCCA
Hormad1	HORMA domain containing 1(Hormad1)	synaptonemal complex assembly	ATAGCATCCAAGCATCCACTTCAC	AGAGACCAGGATAAGAAACGCAG
Kiss1	KiSS-1 metastasis-suppressor(Kiss1)	protein coupled receptor signaling pathway	CGGACCCCAGGAACTCGTTA	GGCATGGCGACGACCTAC
Kit	KIT proto-oncogene receptor tyrosine kinase(Kit)	ovarian follicle development	AGCGTCTTCCGGCACAACGG	AGCGTCTTCCGGCACAACGG
Nr2f2	nuclear receptor subfamily 2, group F, member 2(Nr2f2)	negative regulation of transcription from RNA polymerase II promoter, in utero embryonic development	CAGCGTGCGGAGGAACCTG	GCCTCTCTGTACAGCTTCCCGT
Ntn5	netrin 5(Ntn5)	multicellular organism development, regulation of transcription	AGGCAACAACTACCCCTGCTAC	GAAACCTGGGCGTGCAGAACATA
Piwil1	piwi-like RNA-mediated gene silencing 1(Piwil1)	pachytene, regulation of translation, spermatid development	ACGACGATCAGGGAGTGACC	CACCGTCTCCTGACCTCGTG
Prm1	protamine 1(Prm1)	nucleus organization, spermatid development	GCCAGCACCATGGCCAGATA	GACGGCAGCATCTTCGCCTC
Pttg1	pituitary tumor-transforming gene 1(Pttg1)	regulation of cell growth, DNA repair	GGTGGCGCAGTCTTCGAGTA	ACCAGTGCCCAGCTTCAACC
Smad9	SMAD family member 9(Smad9)	ureteric bud development, regulation of transcription	TGCTCTGAAATTTGGGGACTG	GCCAGAGCCGGCAATATATA
Sox9	SRY (sex determining region Y)-box 9(Sox9)	negative regulation of transcription from RNA polymerase II promoter	AGGCTGTAAAATGCCACTC	CGCTCCGCCTCCTCCACGAA
Spaca1	sperm acrosome associated 1(Spaca1)	acrosome assembly, spermatogenesis	AGCTGAGCTCTATGAGGTGCG	TCCTGTGCCTACCAACCACC
Spo11	SPO11 initiator of meiotic double stranded breaks(Spo11)	leptotene, meiotic DNA double-strand break processing	TTGATTGCTGGCAACTTGAG	CAGATGCTGCATTCCTTGAA
Star	steroidogenic acute regulatory protein(Star)	steroid biosynthetic process, bile acid biosynthetic process, estrogen biosynthetic process, lipid transport	TCTCTGCTTGGTTCTCAACTGG	AAACACCTTGCCCACATCTG
Stk31	serine threonine kinase 31(Stk31)	protein phosphorylation, spermatogenesis	CGTGGAAGCTCGGCGTTTTC	TCTGGGCCCAAAATGTTACTGC
Stra8	stimulated by retinoic acid gene 8(Stra8)	regulation of transcription from RNA polymerase II promoter, male germ-line stem cell asymmetric division, cellular response to retinoic acid	GGAGAAAAAGGCCAGACTCC	CCACGTCAAAAGCATCTTCA
Tdrd1	tudor domain containing 1(Tdrd1)	multicellular organism development, germ cell development	CGTCCGAGGCGAGACATCAG	TTCTGAGAGGGTCCTGGGGC
Thra	thyroid hormone receptor alpha(Thra)	negative regulation of transcription from RNA polymerase II promoter, thyroid hormone mediated signaling pathway	CCCAAGCTGCTGATGAAGGAGAG	ACCTGCGGACCCTGAACAAC
Thrb	thyroid hormone receptor beta(Thrb)	negative regulation of transcription from RNA polymerase II promoter, thyroid hormone mediated signaling pathway	GCTCCAGCGCTCTGATCCGT	CTGGCATTCCCTCTGACCTTCT
Tsga8	testis specific gene A8(Tsga8)	spermatid development, spermatid nucleus differentiation,	TGTTCCGACTTCATCCACCGA	TGGTCTTCGCACCCTTTCCAC
Tssk1	testis-specific serine kinase 1(Tssk1)	protein phosphorylation, multicellular organism development, spermatid development	GATGACGCTGCCGTCCTCAA	TCTGAGGGGGCTTTCTTGCG

ID	MeDIP Forward Primer	MeDIP Reverse Primer
Brdt	CCATAAAGGCGCCAGTCGCT	ATGAAGCAGTGCAGGACCGT
Ddx4	CCTGATGCTATTTGTTGTCCCC	CTTGGAAGGCAGAGGAGG
Hormad1	TTTTGGCGGGAATAGTGGT	AAGCCGAAAATAAACATAATTTAC
Ntn5	CCACGTGACGGTGCCATCTT	CTGAAACCTGGGCGTGCAGA
Tdrd1	TTTGAGCGTCCGAGGCGAG	CTGACCACTCATGCTGGCGT