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The impact of fluoxetine treatment on reproductive organs in male rats following peripubertal PTSD-like stress exposure

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ABSTRACT

Fluoxetine (FLX) is the primary treatment for childhood posttraumatic stress disorder (PTSD); however, it may exacerbate the impact of stress on the development of the male reproductive system. In this study, using a PTSD rat model, we evaluated the long-term effects of peripubertal stress and FLX treatment on the morphophysiological parameters of the testes and epididymis, as well as the levels of hormones that regulate male reproduction. Male 40-day-old rats were subjected to a stress-restress paradigm, followed by 21 days of FLX treatment (10 mg/kg). Behavioral testing confirmed PTSD-like anxiety in stressed rats. The PTSD rats exhibited significant reductions in serum testosterone and follicle-stimulating hormone (FSH) levels irrespective of FLX treatment. Although stress alone did not significantly reduce the sperm count, stressed and FLX-treated rats exhibited decreased sperm numbers, lower epididymis weights, and greater gonadosomatic indices. Histological analyses revealed testicular damage, including peritubular edema, disrupted seminiferous epithelium, and vascular congestion, in both PTSD and PTSD+FLX rats. These effects were more pronounced in the testes of the FLX-treated group, as evidenced by a significant reduction in the numbers of Sertoli cells and spermatogonia and an increase in the number of vacuoles. RNA sequencing revealed only modest gene expression alterations, which were primarily limited to the effects of stress. Thus, our results highlight the detrimental impact of FLX on male reproductive function, particularly in the context of PTSD-induced hormonal dysregulation. Although FLX is widely used for treating PTSD and other psychiatric disorders, its effects on reproductive health underscore the need for a cautious approach when prescribing the drug to adolescents.

1. Introduction

Posttraumatic stress disorder (PTSD) can manifest in children after exposure to even a single traumatic event, and the risk of PTSD significantly increases with repeated or prolonged trauma [1]. Among the pharmacological treatments available for childhood PTSD, fluoxetine, which is a selective serotonin reuptake inhibitor (SSRI), remains one of the most commonly prescribed drugs [2]. The interplay between PTSD and pharmacological intervention with fluoxetine presents a significant challenge concerning its impact on male gonads. PTSD is associated with dysregulation in several neuroendocrine systems, such as the hypothalamic-pituitary-adrenal (HPA) axis, the hypothalamic-pituit ary-thyroid (HPT) axis, and the hypothalamic-pituitary-gonadal (HPG) axis, thereby leading to altered hormone production, including reductions in testosterone, which is essential for maintaining the normal function of the male reproductive system [3]. In conjunction with these effects, SSRIs themselves can negatively affect sexual function and fertility by disrupting the function of the HPA axis, the pancreas, and

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gonads, as well as by dysregulating glucose metabolism [4]. In particular, fluoxetine has been shown to negatively influence male reproductive health [5].Nevertheless, clinical data on the effects of fluoxetine on male reproductive development in children and adolescents are scarce. The majority of human studies have focused on adult patients, wherein fluoxetine has been linked to sexual dysfunction, including reduced libido, delayed ejaculation, erectile dysfunction, and poor semen quality [6–9]. However, the extent to which these findings can be extrapolated to younger populations is unclear, as the developing reproductive system may be more vulnerable to drug-induced disruptions. One clinical concern is that SSRIs such as fluoxetine may affect gonadal hormone levels during puberty, which is a time period characterized by rapid hormonal changes that are crucial for reproductive maturation. Although direct clinical evidence of the impact of fluoxetine on adolescent male reproductive function is limited, the reported effects of this drug on the HPA and HPG axes [10] raise questions about its potential to interfere with normal pubertal development. Furthermore, some reports have noted delayed puberty in adolescent males receiving SSRI treatment. The authors of a previous study concluded that such findings could be related to the impact of SSRIs on the HPG axis, thus resulting in lower levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and sex hormones, although these findings remain anecdotal and require further investigation [11].

Moreover, animal studies have provided valuable insights, with findings suggesting possible adverse outcomes when the drug is administered during sensitive developmental stages. Research involving juvenile rats has indicated that fluoxetine exposure may interfere with normal testicular function and spermatogenesis. For example, maternal exposure to fluoxetine during lactation in mice has been associated with significant reductions in offspring body and testis weights, all sperm parameters, the tubular diameter, and the epithelial height of the seminiferous tubules, as well as the number of Leydig cells, possibly resulting from disruptions in the HPG axis, which plays a central role in regulating reproductive development [12]. Testosterone production is linked to LH and FSH, both of which are regulated by gonadotropin-releasing hormone [13]. Fluoxetine elevates extracellular serotonin (5-HT) levels in the brain; correspondingly, 5-HT modulates the release of gonadotropin-releasing hormone (GnRH) within the HPG axis [14], thereby influencing testosterone levels [15]. Male rats treated with fluoxetine during adolescence exhibit altered LH, FSH, progesterone, and testosterone serum concentrations [15]. These findings underscore the potential for fluoxetine to exert harmful effects on the developing male reproductive system, particularly during the critical window of adolescence. Another study revealed that rats exposed to fluoxetine exhibited increased oxidative stress in testicular tissues, thereby indicating that fluoxetine may also contribute to long-term reproductive dysfunction via mechanisms involving oxidative damage [16]. Furthermore, Leydig cells may represent a target of fluoxetine in adult rat testes via impairments in the integrity of peritubular myoid cells and Sertoli cells, as well as disturbances in spermatogenesis [17].

Thus, multiple lines of evidence indicate the possibility of the combined burden of PTSD and fluoxetine treatment on the developing male reproductive system. Based on these concerns, our study aimed to investigate the combined effects of PTSD induced at peripubertal age and fluoxetine treatment during the pubertal period on hormones that regulate the male reproductive system and testicular parameters in adulthood. By employing an animal model of PTSD, we sought to better understand the potential risks associated with fluoxetine use in adolescents.

2. Materials and methods

2.1. Animals and experimental design

This study was performed in male Wistar rats from the colony of the SI “Institute of Pharmacology and Toxicology NAMS of Ukraine”. The original breeding pairs were purchased from Charles River Laboratories (France). This study was designed and reported following the ARRIVE guidelines [18], performed according to the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, and approved by the Institutional Animal Care and Use Committee (Ethical Nr. 010522).

Males of initial ages of 40 days (weighing 120–140 g) were randomly selected from different parent generations (40 postnatal days (PND) in rats is comparable to the human peripubertal age [19]). The animals were housed at 3 animals per cage and maintained at a controlled temperature (from 22 °C to 24 °C), with a relative humidity of 40–70 %, lighting (12 h light-dark cycle), and free access to a standard pellet feed diet (LLC NTV "F.U.D.", Ukraine) and tap water.

The rats were randomly divided into the following 3 groups: the control (n = 26), PTSD (n = 26), and PTSD+fluoxetine (FLX) groups (n = 26). Ten rats from each group were used for testes and epididymis histological analyses, and 14 rats were used for hormone evaluation (samples exhibiting hemolysis were excluded). All of the groups were used for behavioral tests, morphometric analysis of gonads and determination of the sperm count.

Rats aged 40 days were subjected to the combined stress-restress paradigm to simulate PTSD [20,21]. The animals were subjected to intense combined stress, which included 2 h of restraint in a Plexiglas tube (14 ×26 cm), followed by 20 min of forced swimming in a water tank (64.5 cm in water depth; 28.9 cm in diameter) at 24–26 °C. After gentle drying and a 15-minute resting period, the animals were exposed to ether vapor until loss of consciousness. The reactive pathological state was triggered seven days after the initial combined stress by a restress stimulus, which involved 30 min of restraint. The control rats remained undisturbed in their home cages in another room.

Beginning at 24 h after the restress stimulus, rats in the PTSD+FLX group were administered fluoxetine. A pharmaceutical preparation of fluoxetine (Flutisal, hard capsules, Kusum, India) was used. The preparation was suspended in 1 % starch gel (w/v) and intragastrically administered via gavage. Fresh suspensions were prepared on a daily basis. The utilized dose of fluoxetine was based on the weights of the rats on the first day of treatment and was corrected on a weekly basis according to weight changes. The control and PTSD groups received only starch gel at the corresponding volume (2.5 ml/kg).

Fluoxetine was administered at a dosage of 10 mg/kg per day for 21 days. In humans, fluoxetine prescriptions range from 20 to 80 mg/day [22,23]. Assuming that the human weight in the most conservative approach is 50 kg, this dosage of fluoxetine would correspond to approximately 0.4–1.6 mg/kg. Thus, when considering the allometric scaling method [24], the dose of 10 mg/kg approximately corresponds to the maximal dose that could ever be used in the clinical setting for children and adolescents [25,26]. Moreover, this dose has been demonstrated to produce clinically relevant fluoxetine plasma concentrations in rats [27].

The complete experimental timeline and detailed procedures are presented in Fig. 1.

2.2. Behavioral evaluation

The elevated plus maze test was performed for all of the groups at 1 day after the end of fluoxetine administration. This test has been previously validated by Pellow et al. [28] using behavioral, physiological, and pharmacological measures.The apparatus comprised two open arms (50 × 10 cm) that were situated across from each other and also situated perpendicular to two closed arms (50 × 10 cm) with a central platform (10 × 10 cm); moreover, 40 cm high opaque walls on two sides surrounded the closed arms, with the entire apparatus situated 70 cm above the floor. After 30 min of habituation to the testing room, the rats were placed on the central platform of the maze facing an open arm and were allowed to explore the maze for 5 min. The behaviors of the rats were tracked, recorded, and analyzed by using the EthoVision XT15

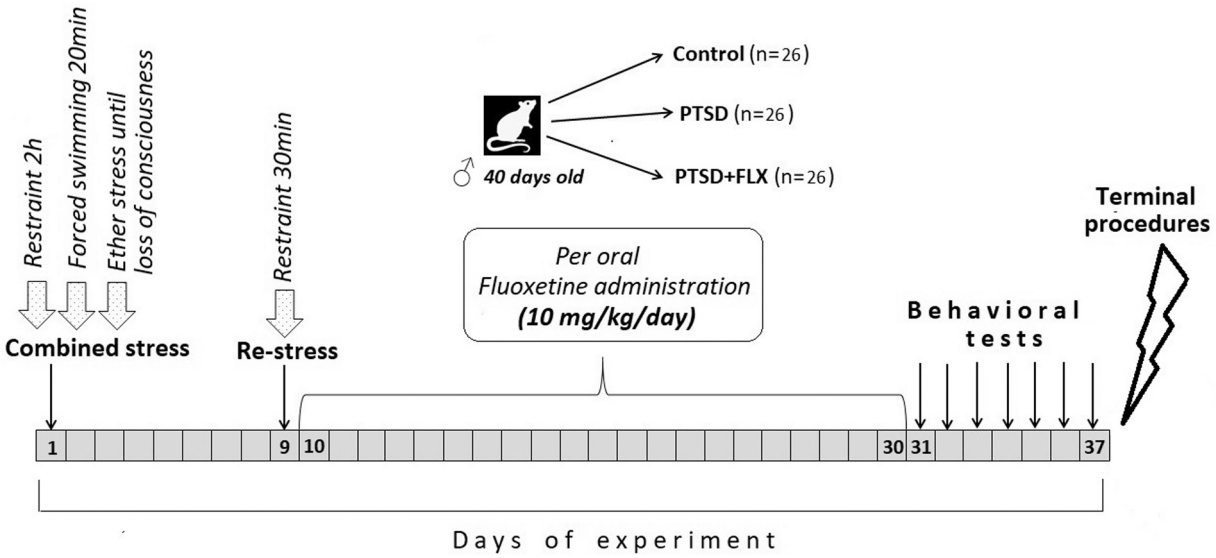

Fig. 1. The general design of the experiment was as follows. Male rats were divided into 3 groups, including the control, PTSD, and PTSD+FLX groups. The PTSD and PTSD+FLX groups were subjected to intense combined stress, which included 2 h of restraint, followed by 20 min of forced swimming and ether stress until loss of consciousness. Seven days after the initiation of the stress procedure, the animals were subjected to 30 min of restraint (the restress stimulus) to model the effects of PTSD. The FLX treatment was initiated 24 h after the restress stimulus and lasted for 21 days. From day 31 to day 37, behavioral tests were performed to estimate PTSD-like symptoms. Afterwards, euthanasia and gonad investigations were conducted.

tracking system (Noldus Information Technology, Wageningen, The Netherlands). The behavioral indices included the distance traveled (cm), the number of entries into the closed and open arms, the time spent in the closed and open arms (s), and the anxiety index, which was calculated as follows: anxiety index = 1 − [([time spent in the open arms/total time spent in the maze] + [number of entries into the open arms/total activity in the maze])/2] [29].

2.3. Hormones regulating the male reproductive system

Immediately before the rats were euthanized, they were anesthetized with diethyl ether, and blood from the femoral vein was collected into polypropylene test tubes, in which the silicone gel and clot activator were separated. In all of the animals, blood collection was performed between 11:00–12:00 a.m. Moreover, serum was separated via centrifugation at 3000 rpm for 15 min, after which the samples were kept frozen at −70 °C until being utilized. The testosterone (T) concentration was assayed by chemiluminescence (IMMULITE® 2000 Immunoassay System, Siemens Healthcare GmbH, Germany). LH and FSH levels were measured using Rat LH and Rat FSH ELISA kits (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions.

2.4. Morphometric parameters of the gonads and sperm number

After 6 days of behavioral tests (which included the elevated plus maze), the rats were subjected to mild diethyl ether (80 μL/L) narcosis for approximately 5 min and subsequently decapitated. The testes and epididymis were removed for examination. A precision electronic balance was used to evaluate the testicular and epididymal weights. The testes were also measured using an electronic caliper, and their volumes were calculated using the following formula for the calculation of a solid ellipsoid triaxial body, which was calculated as follows:4πabc/3, where a π is equal to 3.14, a = half the heigh, b = half the width, and c = half the length [30].

Both testes and both epididymides of each rat were measured, and the mean values were calculated. The mean testicular and body weights were used to derive the gonadosomatic index using the following formula: gonadosomatic index = testicular weight/body weight × 100 [31].

The distal cauda segments from the left epididymis were separated. Each cauda was cut and minced in 2 ml of normal saline [32]. An aliquot of the epididymal suspension was subsequently used for sperm count analysis via a Makler chamber under a light microscope (200x).

2.5. Histological analyses of the testis and epididymis

The right testicle and epididymis were used for histological evaluations. Histological slide processing and examination were conducted as previously described [33]. Three slides were prepared for each animal.

The quantitative parameters of spermatogenesis were evaluated based on the estimated number of cell layers, types of cells, and presence of late spermatids in the seminiferous tubules. The evaluation grades (corresponding to spermatogenesis stages) were as follows: 1 – only spermatogonia present; 2 – spermatogonia and spermatocytes present; 3 – spermatogonia, spermatocytes, and round (early) spermatids present with < 5 late spermatids observed per tubule; and 4 – spermatogonia, spermatocytes, and round spermatids present with < 25 late spermatids observed per tubule [34]. The spermatogenesis index was then calculated by dividing the sum of these grades by the number of examined seminiferous tubules, totaling 200 tubules per testis for each animal. Additionally, the number of Sertoli cells per cross-section of seminiferous tubules was determined by analyzing the same 200 tubules per testis per animal.

The quantification of Leydig cells was conducted utilizing a Leica DM LS microscope, following the calibration of the 40x objective lens with an ocular grid. The cells were enumerated in all squares of the grid that overlapped with the tubules while excluding those positioned along the upper and right borders of the grid. Counting was performed in five randomly chosen areas on each slide. A total of three slides were analyzed for each animal, and the mean Leydig cell count per mm^2 was subsequently calculated for each individual and each group.

Cell exfoliation (which was characterized by the shedding of

epithelial components), along with the desquamation of the epithelium from the basal membrane of the tubule, was also noted. Additionally, the presence of cell-free areas (identified as vacuoles) served as indicators of impaired spermatogenesis.

2.6. RNA Extraction and RNA-seq

Testes were dissected and stored frozen at −20 °C in RNAlater solution (Thermo Fisher Scientific) for RNA stabilization. Five samples per group were randomly selected for RNA extraction. Approximately 50 mg of tissue per sample was homogenized in 1000 μl of TRIzol™ Reagent (Thermo Fisher Scientific) using a TissueLyser (QIAGEN, Germany), and total RNA was extracted according to the manufacturer's protocol. Briefly, after the samples were homogenized with TRIzol™ Reagent, 200 μl of chloroform was added. The samples were then vortexed for 30 s at room temperature, and the homogenate was allowed to separate on ice into a clear upper aqueous layer (containing RNA), as well as into interphase and red lower organic layers (containing the DNA and proteins). The samples were subsequently centrifuged (13000 × g at 40 °C for 20 min), the upper aqueous layer was collected (450 μl), and the RNA was precipitated with 450 μl of isopropanol. After centrifugation (13000 × g at 40 °C for 20 min), the RNA pellets were washed once with 1000 μl of ice-cold 70 % ethanol to remove impurities and subsequently resuspended in 100 μl of RNAse-free water. The integrity and concentration of the extracted RNA were assessed using an Agilent RNA 6000 Nano Kit on a 2100 Bioanalyzer (Agilent, USA). Based on RNA integrity number (RIN > 7) values, all 15 samples were chosen for RNA sequencing. RNA sequencing was performed as an external service by Novogene Ltd. (UK). Briefly, poly(A) RNA was isolated from total RNA samples by the addition of oligo(dT) beads. Additionally, cDNA libraries were prepared on a template of randomly fragmented mRNA using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs, USA). Complete cDNA libraries consisting of fragments 250–300 base pairs in length that were acquired from the samples were subjected to sequencing on a NovaSeq 6000 with the following parameters: PE 150 (paired ends) and 40 M clean reads, which yielded more than 12 Gb of raw data per sample.

The RNA-seq data were processed using the nf-core/rnaseq pipeline v3.14.0 [35] of the nf-core collection of workflows [36] with default parameters. Within the pipeline, FASTQ files were checked for quality and aligned to the rat reference genome mRatBN7.2.

2.7. Statistical analysis

The obtained numerical data were processed with STATISTICA v. 13 (StatSoft, Inc., Tulsa, OK, US). The normality of the distribution of the continuous variables was checked by the Shapiro-Wilk test. Continuous variables with a normal distribution are presented as the mean ± standard error of the mean (M ± SEM) and were compared using one-way ANOVA, followed by the post hoc Tukey test. Variables with a non-normal distribution are presented as the median (Me) and interquartile range (IQR) and were compared using the Kruskal-Wallis test, followed by the Dunn's post hoc test.

The effects of PTSD and treatment on weight gain were assessed using mixed model analysis of variance (type III ANOVA) with correction for sphericity, followed by the Tukey's HSD test. Analyses were performed using R and the implementation of ANOVA in the car library with the afexaddon. Tukey's HSD was assessed using the emmeans library.

The statistical significance threshold was set at $p < 0.05$.

Differential gene expression analysis of the RNA-seq data was performed using the R package edgeR (v3.38.4). P values were adjusted for multiple testing using the Benjamini-Hochberg method to control for the false discovery rate. Due to the fact that this method revealed only one regulated gene, we further applied a less stringent differential expression criterion (a >1.5-fold change at nominal $p < 0.001$).

3. Results

3.1. Evaluation of anxiety-like behavior

We assessed behavioral changes mimicking PTSD symptoms using the elevated plus maze test. In the PTSD group, the cumulative duration of time spent in the closed arms (s) was more than 20 % longer than that in the control group ($H(2) = 6.869$, $p = 0.032$) (Fig. 2A). Moreover, the time spent in the open arms tended to be shorter in the PTSD group than that in the control group (Fig. 2B). A similar trend was observed in the number of entries into the open arms; however, this difference was not statistically significant (Figs. 2C and 2D). The results revealed that the total distance traveled by the rats in the PTSD and PTSD+FLX groups during the 5-minute test did not significantly differ from that of the control group (Fig. 2E). Taken together, the results demonstrated that there was a significant effect of the PTSD procedure on the anxiety index based on the combination of measures from the elevated plus maze (Fig. 2E, $H(2) = 5.978$, $p = 0.050$).

Moreover, there were no significant differences observed between the PTSD+FLX group and the control or PTSD groups (Fig. 2A-2F).

3.2. Hormones regulating the male reproductive system

Compared with the control group, both the PTSD and PTSD+FLX groups exhibited a significant decrease in serum testosterone levels to approximately half of the total serum testosterone level ($F(2, 36) = 4.622$, $p = 0.016$) (Table 1). The decrease in testosterone was accompanied by an approximately 20 % decrease in FSH levels in the same groups ($F(2, 36) = 5.4896$, $p = 0.008$) (Table 1). The LH concentration in the serum remained unchanged in both stressed groups ($F(2, 38) = 0.25824$, $p = 0.77375$) (Table 1).

3.3. Body weight, morphometric parameters of gonads, and sperm number

There was a significantly slower weight gain observed in the fluoxetine-treated PTSD group than in the control group (Fig. 3, $F(76) = 3.989$, $p = 0.001$), whereas a similar trend was not statistically significant in the PTSD group ($F(76) = 1.610$, $p = 0.248$).

Compared with those of the control and PTSD groups, the morphometric parameters of the reproductive organs revealed that exposure to both PTSD and fluoxetine did not affect testis size ($H(2) = 1.578$, $p = 0.454$) or absolute testis weight ($H(2) = 1.513$, $p = 0.469$) (Fig. 4). Conversely, a decrease in the absolute weight of the epididymis ($F(2, 75) = 6.153$, $p = 0.003$) and an increase in the relative testis–weight gonadosomatic index ($F(2, 75) = 5.364$, $p = 0.007$) were observed in the group that received fluoxetine treatment.

PTSD had no significant effect on the sperm count, whereas the number of spermatozoa from the cauda epididymis was reduced in the animals with PTSD that received fluoxetine treatment ($H(2) = 7.087$, $p = 0.029$) (Fig. 4).

3.4. Histological analyses of the testis and epididymis

The testicular tubules of the control group exhibited a normal histology, with germ cells in all stages of differentiation (although not all of them were observed in each tubule), a tubular lumina with mature sperm, and a typical interstitial compartment with Leydig cells and intact blood vessels (Fig. 5A and 5B; 6A).

Conversely, the testes of the rats in the PTSD and PTSD+FLX groups demonstrated multiple small areas with noticeable peritubular tissue edema and disruptions in the seminiferous epithelium. In some of the assessed sections, the edema foci led to a marked expansion of the peritubular spaces and compromised the integrity of the seminiferous tubule walls due to the emergence of edema beneath the basal membranes of the tubules (Fig. 5D). Detachment of the seminiferous

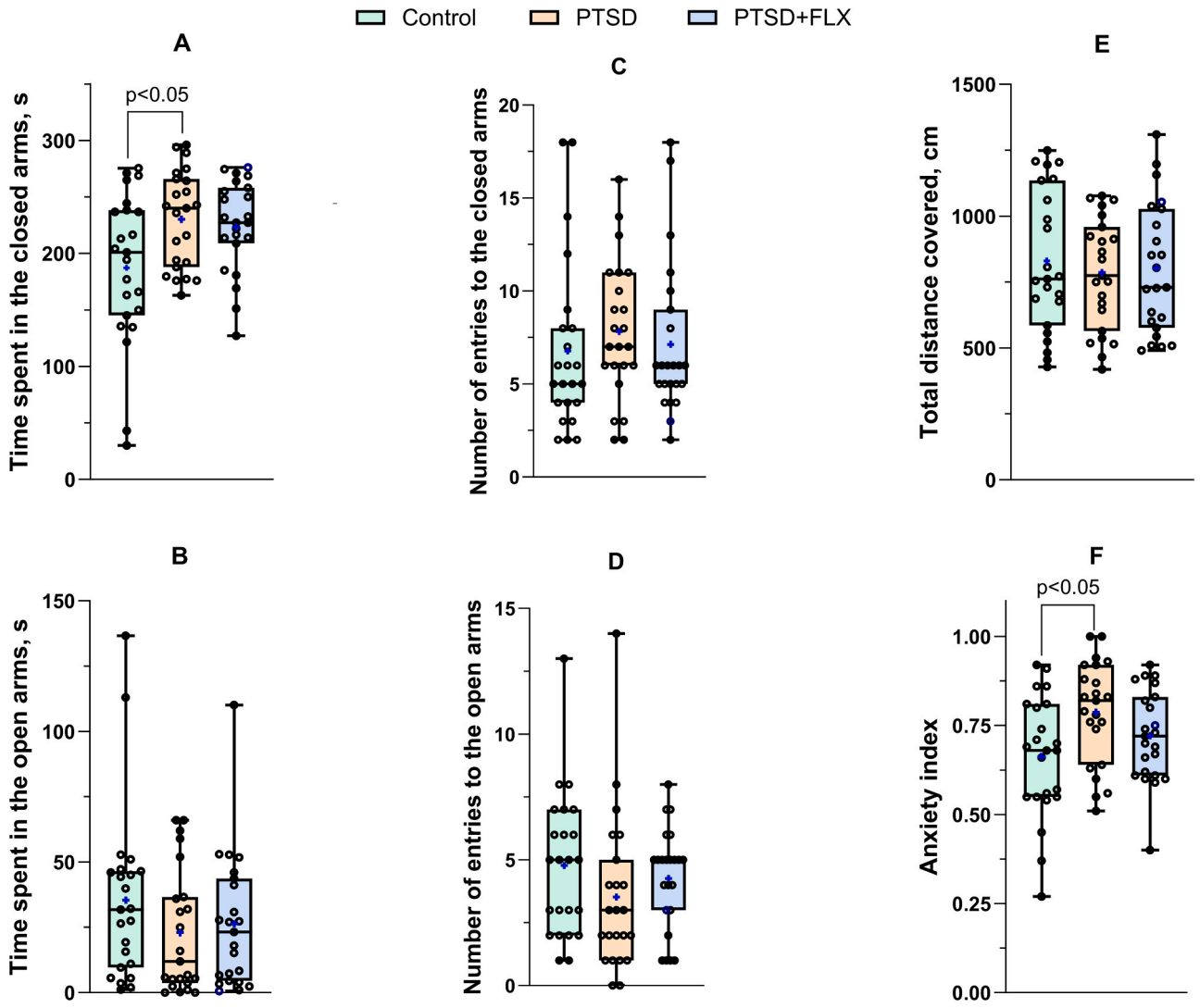

Fig. 2. Effects of PTSD simulation and fluoxetine treatment on the anxiety-like behaviors of rats in the elevated plus maze (A - time spent in the closed arms; B - time spent in the open arms; C - number of entries into the closed arms; D - number of entries into the open arms; E - total distance traveled; F - anxiety index). The data are presented in a box-whisker plot, where the box represents the 1st and 3rd quartiles, the middle line represents the median, and the "+ " represents the mean. The whiskers represent the minimum and maximum values in the group. The indicated *p* values were obtained by using the Kruskal-Wallis test, followed by the Dunn's post hoc test.

Table 1
Serum hormone levels in rats subjected to PTSD simulation and fluoxetine treatment at PND 77.

Parameters	Groups		
	control	PTSD	PTSD + FLX
Testosterone, nmol/L	15.67 ± 3	8.15 ± 1.25*	8.34 ± 1.16*
FSH, ng/ml	137.14 ± 6.34	112.38 ± 7.28*	108.56 ± 5.77*
LH, IU/L	38.7 ± 3.54	37.28 ± 2.85	35.55 ± 2.97

Data are presented as mean ± SEM
* $p < 0.05$ compared with the control (one-way ANOVA, followed by a post hoc Tukey test).

epithelium from the basement membrane was observed in significantly more seminiferous tubules in the fluoxetine-treated group than in the other groups ($H(2) = 12.557$, $p = 0.002$) (Fig. 5C). Edema frequently coincided with significant dilation of lumens and congestion in small-caliber arteries, veins, and blood capillaries surrounding clusters of interstitial endocrinocytes. Additionally, small-caliber arteries often exhibited wall thickening resulting from edema of smooth muscle cells in the inner vessel lining and connective tissue in the outer vessel lining, which was occasionally accompanied by hyalinosis of collagen fibers. The arterial walls also demonstrated irregular basement membrane thickening, small foci of subendothelial edema, and pronounced corrugation of the internal elastic membrane (Fig. 5E).

Edema and disorganization of collagen fibers in the peritubular connective tissue were accompanied by an irregular arrangement of interstitial endocrinocytes (Leydig cells) and compartmental cells. Dystrophic changes characterized by small foci of pericellular edema were also evident in the interstitial endocrinocytes themselves (Fig. 5F and 5G). In addition, compared with the control group, the PTSD group exhibited a decreased number of Leydig cells ($F(2, 25) = 3.756$, $p = 0.0375$) (Fig. 5H).

In addition to the observed edema, we identified other alterations in the testicular tissue across both experimental groups. Notably, small apoptotic bodies were frequently observed in the basal areas of the seminiferous tubules containing Sertoli cells (Fig. 6B). The number of these cells was much greater in PTSD animals than in control animals, thereby indicating the apoptosis of sustenocytes or spermatogonia. The quantitative analysis revealed that the number of spermatogonia was

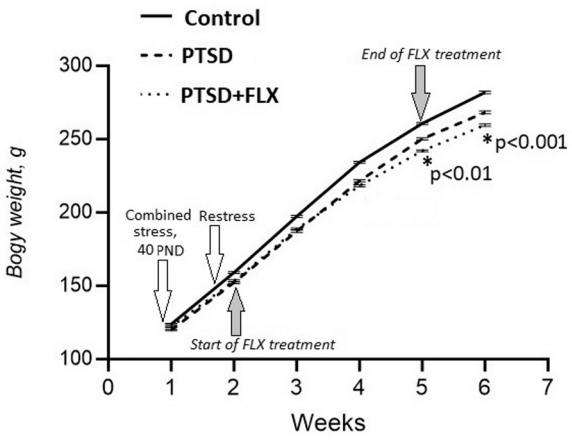

Fig. 3. Dynamics of body weight changes in rats with PTSD simulation and fluoxetine treatment. The data are presented as the means ± SEMs. The indicated p values were obtained using mixed model analysis of variance (type III ANOVA) with correction for sphericity, followed by the Tukey's HSD test. * - statistically significant difference between the PTSD+FLX group and the control group.

significantly decreased in both the PTSD and PTSD+FLX groups ($F(2, 27) = 157.24$, $p = 0.00000$), with a substantially greater reduction (almost 20 %) being observed in the PTSD+FLX group (Fig. 6C). Moreover, compared with those in the control and PTSD groups, the number of Sertoli cells in the fluoxetine-treated group was prominently decreased ($F(2, 26) = 71.273$, $p = 0.00000$) (Fig. 6D).

Basophilic bodies were detected within the adluminal zone of the seminiferous tubules (Fig. 6E and 6F), which suggests degeneration of spermatogenic epithelial cells during the later stages of development. Despite these observed alterations, the germ cell layers were present in normal numbers, as indicated by the stable spermatogenesis index ($F(2, 27) = 1.467$, $p = 0.249$) (Fig. 6G); moreover, the number of cells in the 12th stage of spermatogenesis (characterized by meiosis of the primary spermatocytes) remained unchanged ($F(2, 27) = 1.669$, $p = 0.207$) (Fig. 6H).

Intraepithelial vacuoles of varying sizes were present in some seminiferous tubules in all of the animal groups; however, their number was significantly greater in the PTSD and PTSD+FLX groups ($F(2, 27) = 7.139$, $p = 0.003$) (Fig. 6I and 6K). Additionally, spontaneous exfoliation of the germinal epithelium into the lumens of some seminiferous tubules was observed in all of the groups. The number of such tubules with exfoliated cells tended to increase in the PTSD+FLX group (Fig. 6J and 6L); however, the difference was not statistically significant ($F(2, 27) = 0.177$, $p = 0.838$).

There were considerably fewer changes detected in the epididymides of the rats from the PTSD and PTSD+FLX groups. Compared with the morphological pattern of the epididymis in the control group (Fig. 7A), small foci of peritubular edema accompanied by slight narrowing of the lumens of the adjacent tubules were often observed in the connective tissue surrounding the ejaculatory ducts (tubules) in both experimental groups (Fig. 7B). Additionally, in the PTSD+FLX group, we observed changes characteristic of blood circulation disorders in the epididymis (such as hyperemia of small-caliber arteries and veins), accompanied by disturbed blood vessel wall permeability (including focal points of perivascular edema, thickening of the blood vessel wall, edema of smooth muscle cells, subendothelial edema foci, and internal elastic membrane corrugation). Nevertheless, despite moderate dystrophic changes being observed, all of the ejaculatory duct lumens were densely filled with fully formed spermatozoa and a small amount of light fluid, which is typical for the normal process of spermatozoa maturation.

3.5. Effects of PTSD and fluoxetine on gene expression in the testes

To further assess the effects of stress and fluoxetine treatment on testicular development, RNA-seq analysis of gene expression was performed ($n = 5$ per experimental group). Analysis using the edgeR, followed by p value adjustment for multiple testing (FDR<10 %), revealed that only one gene (ENSRNOG00000069801) was significantly regulated in the PTSD group, whereas no genes were regulated by fluoxetine. Therefore, we applied a less stringent differential expression criterion to filter for potentially regulated genes in our experimental paradigm. We detected 33 transcripts with a > 1.5-fold change in abundance (nominal $p < 0.001$; Fig. 8) in the PTSD group. Among these genes, 17 transcripts, including the immediate-early gene *Egr1* and the metabotropic glutamate receptor 4 (*Grm4*) gene, exhibited stress-induced downregulation. The upregulated transcripts included myosin light chain 1 (*Myl1*) and six long noncoding RNAs (LOC103692103, ENSRNOG00000062562, ENSRNOG00000070554, LOC108348724, AABR07064867.2, and LOC102549654).

None of the 33 transcripts met the significance threshold for differential expression in either the control versus fluoxetine-treated or stress versus fluoxetine-treated comparisons. Gene ontology analysis using Enrichr [37] utilizing all of the available annotation sources yielded no significantly overrepresented terms. Together, these results suggest that although stress induced detectable morphological changes, its impact on testicular gene expression was relatively modest.

4. Discussion

Our findings demonstrate that PTSD modeling during the peripubertal period in male rats causes structural and functional alterations in the reproductive system at later points in time, with additional impairments being observed following fluoxetine treatment. Both the PTSD and PTSD+FLX groups exhibited marked reductions in serum testosterone and FSH levels, which were accompanied by histological abnormalities in the testes, including vascular disturbances, interstitial edema, and structural disorganization of the seminiferous tubules. Fluoxetine did not normalize these stress-induced changes; rather, in some cases (such as Sertoli cell depletion and reduced sperm count), fluoxetine further exacerbated reproductive impairments. Despite these alterations, the spermatogenesis indices remained stable, thereby suggesting a degree of compensatory maintenance within the seminiferous epithelium. Collectively, these results indicate that peripubertal PTSD and fluoxetine (when administered during critical developmental stages) may contribute to future testicular dysfunction.

The results of the elevated plus maze demonstrated increased anxiety in PTSD rats (as evidenced by the increased time spent in the closed arms and the anxiety index), thus suggesting the accuracy of the utilized model. Fluoxetine administration to PTSD-exposed rats did not significantly improve performance in the elevated plus maze test. This outcome suggests the limited efficacy of fluoxetine in mitigating PTSD-like behaviors in this experimental setup, thus potentially reflecting the challenges regarding this drug's efficacy in addressing PTSD symptoms. In contrast to some previous reports [38,39], our findings are consistent with the suggestion that the pharmacological treatment of PTSD may offer only limited efficacy in certain contexts [40].

In conjunction with previous studies, we observed a tendency of slower weight gain in the PTSD group [41–43].In the group receiving fluoxetine treatment, the weekly average cumulative body weight gain began to significantly decrease at the end of the treatment period and remained lower than that in the control group until the end of the experiment. This finding is expected based on previous studies regarding the effects of fluoxetine on body weight regulation in both animals and humans. Previous studies in normal and obese rodents have demonstrated that chronic treatment with different doses of fluoxetine reduced food consumption, which led to decreases in body weight and body mass index [44,45]. In children aged 7–17 years, the impact on growth

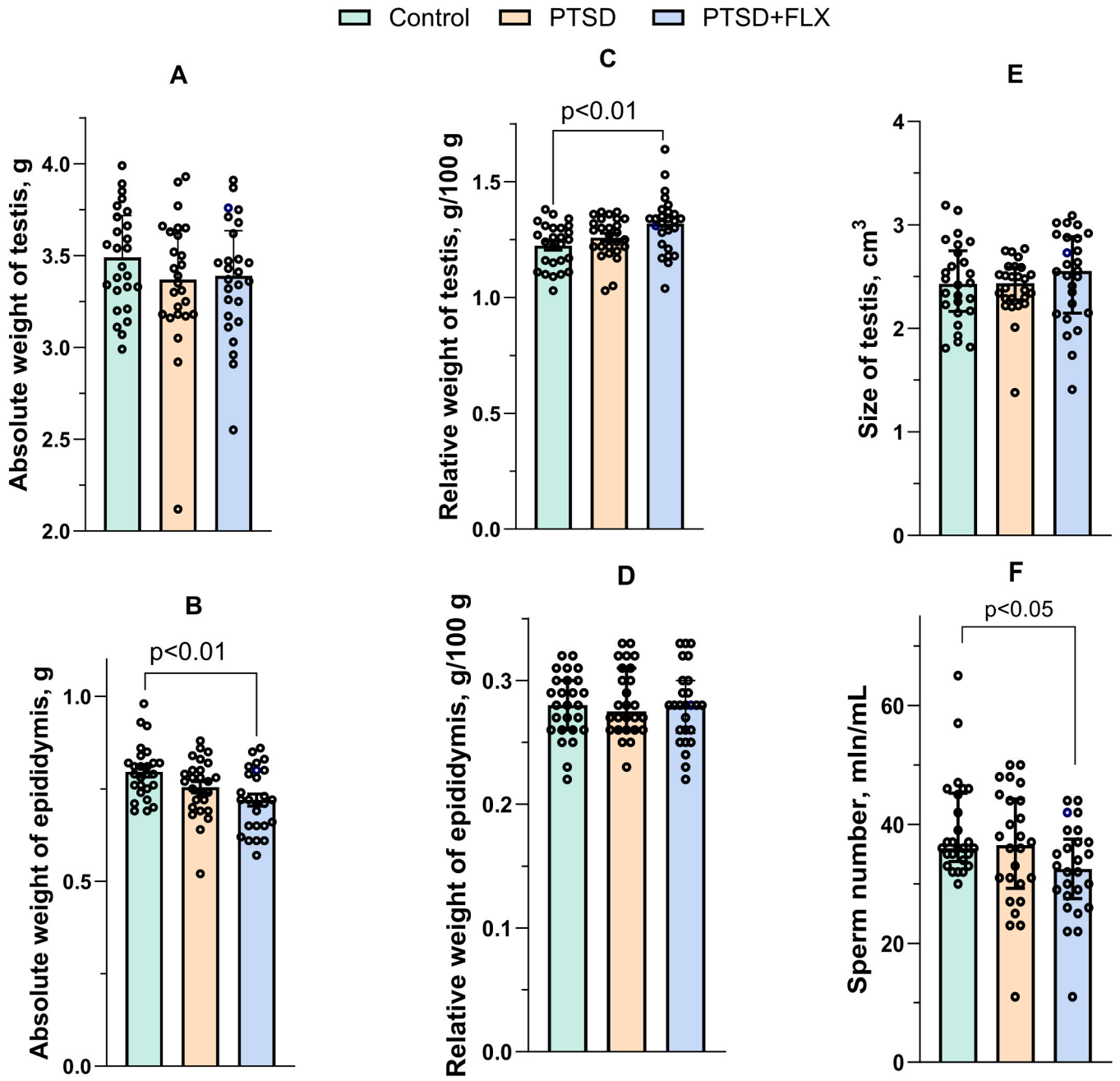

Fig. 4. Morphometric parameters of reproductive organs and sperm number at PND 77 from rats subjected to PTSD simulation and fluoxetine treatment (A - absolute weight of the testis; B - absolute weight of the epididymis; C - relative weight of the testis; D - relative weight of the epididymis; E - size of the testis; F - sperm number). The vertical bar graphs represent the means±SEMs for variables with a normal distribution or medians with interquartile ranges for variables with a nonnormal distribution. The indicated *p* values were obtained using one-way ANOVA, followed by a post hoc Tukey test or Kruskal-Wallis test, as well as by a post hoc Dunn's test.

emerged as a significant outcome after 19 or 36weeksof fluoxetine administration [46,47]. Moreover, this drug has been proposed to affect energy balance via two mechanisms: its ability to stimulate the expression of brain-derived neurotrophic factor (BDNF) in the hypothalamus and its ability to improve central leptin sensitivity. Notably, these mechanisms are interconnected, with energy output being the primary factor influenced by this interaction [48].

Our findings demonstrate that PTSD simulation during the peripubertal period affects the HPG axis. Specifically, we observed reduced FSH levels, whereas LH levels remained unchanged. Additionally, we observed a decrease in testosterone levels, thereby suggesting impaired gonadal function in the PTSD group. As indicated in the review by Raise-Abdullahi et al. (2023), PTSD is associated with changes in several neuroendocrine systems beyond the HPA axis, including abnormalities in monoamine signaling, as well as changes in the HPT and HPG axes [49]. The function of the HPG axis in PTSD has not received as much research attention as that of the HPA axis. In human studies, direct research on FSH and LH levels in male PTSD patients is limited.Moreover, clinical reports concerning testosterone levels in males with PTSD are inconsistent, demonstrating either increases, decreases or a lack of change in these levels [50–52]. In adult patients, this scenario may be due to a number of factors, such as comorbidities, concomitant medications, and various types of addiction.

FSH and testosterone levels were also observed to be lower in the

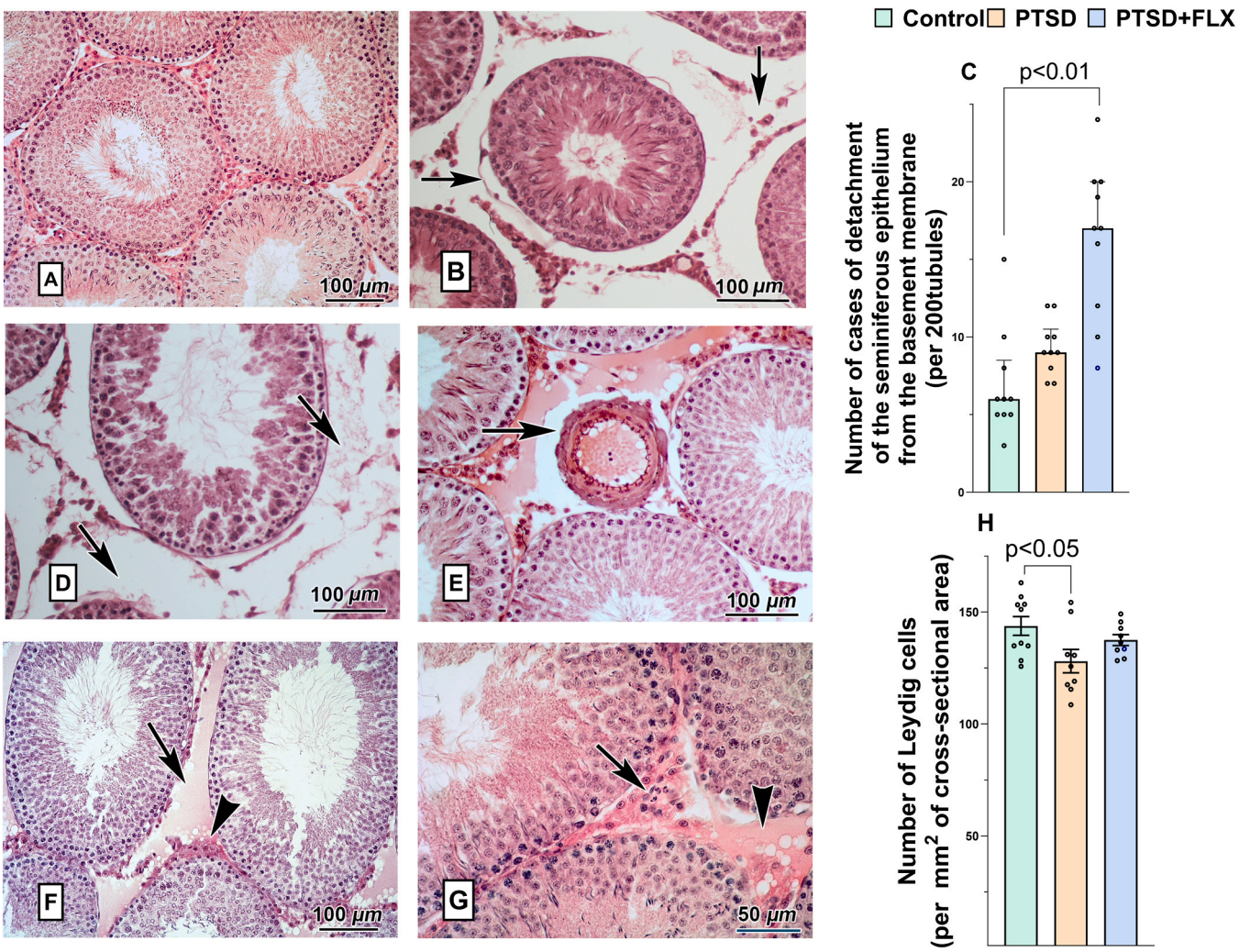

Fig. 5. Examples of edema present in the testes of PTSD model and FLX-treated rats (A - unchanged histological structure of control group rat testes; B and D - PTSD and PTSD+FLX: peritubular edema, expansion of peritubular spaces, disorganization of compartment-shaped cells, and detachment of the seminiferous epithelium from the basement membrane (arrows); C - quantitative data: number of cases of detachment of the seminiferous epithelium from the basement membrane; E - PTSD: expansion of the lumen and congestion of a small-caliber artery with a significantly thickened wall due to edema of smooth muscle cells, as well as edema and hyalinosis of collagen fibers of the outer shell and pronounced corrugation of the internal elastic membrane of the artery wall (arrow); F - PTSD: expansion of peritubular spaces and their filling with light, acidophilic liquid (arrow), along with disorganization of interstitial endocrinocytes (arrowhead); G - PTSD+FLX: intense pericellular edema (arrowhead) and disorganization of endocrinocytes (arrow); H - quantitative data: Leydig cell number). Testis cross-sections: hematoxylin and eosin staining. Quantitative data: the graphs represent the medians with interquartile ranges for variables with a nonnormal distribution (C) or means±SEMs for variables with a normal distribution (H). The indicated *p* values were obtained using one-way ANOVA, followed by a post hoc Tukey test or Kruskal-Wallis test, as well as by a post hoc Dunn's test.

PTSD+FLX group than in the control group. We expected PTSD-induced hormonal alterations to be aggravated by fluoxetine, due to the fact that the drug exerts deleterious effects on glucose metabolism, sexual function, and fertility by interfering with the HPG and HPA axes and exacerbating reproductive hormonal imbalances [4].

Due to the fact that fluoxetine is a serotonin reuptake inhibitor, serotonin-related effects could contribute to the modulation of the effects of PTSD on male gonads.

It has been demonstrated that the decline in FSH levels may be attributed to elevated levels of 5-HT in the brain, which affect the secretion of gonadotropin-releasing hormone (GnRH) [4]. Beyond central modulation, fluoxetine may exert direct effects on testicular steroidogenesis, due to the fact that 5-HT acts on 5-HT2 receptors in Leydig cells that are distinct from those in the brain [53]. A recently proposed hypothesis suggests that 5-HT and its components represent an as-yet-unrecognized local inhibitory control mechanism related to blood vessels, steroidogenesis, and spermatogenesis in testicular function [54].

Research has also indicated that fluoxetine treatment can alter the activity of 3ß-hydroxysteroid dehydrogenase (3ß-HSD) and 17ß-hydroxysteroid dehydrogenase (17ß-HSD) [55]; moreover, these enzymes are involved in the conversion of 3β-hydroxysteroids into 3-keto--steroids [56] and in the conversion of delta 4 androstenedione into testosterone [57], respectively.The effect of fluoxetine on 3ß-HSD and 17ß-HSD suggests a potential mechanism by which it cans directly influence testosterone synthesis within the testes.

Contrary to our expectations, testosterone and FSH levels in the fluoxetine-treated group were similar to those in the PTSD group. These findings suggest that fluoxetine administration does notfurther exacerbate the hormonal disruptions induced by PTSD. In any case, PTSD and fluoxetine exposure during early developmental stages could be implicated in persistent hormonal imbalances, which most likely occur due to interactions with HPG axis maturation. Overall, our study corroborates the current evidence concerning the impacts of fluoxetine on reproductive hormones. However, such effects can vary depending on the dosage, timing of administration, age, and other conditions of the

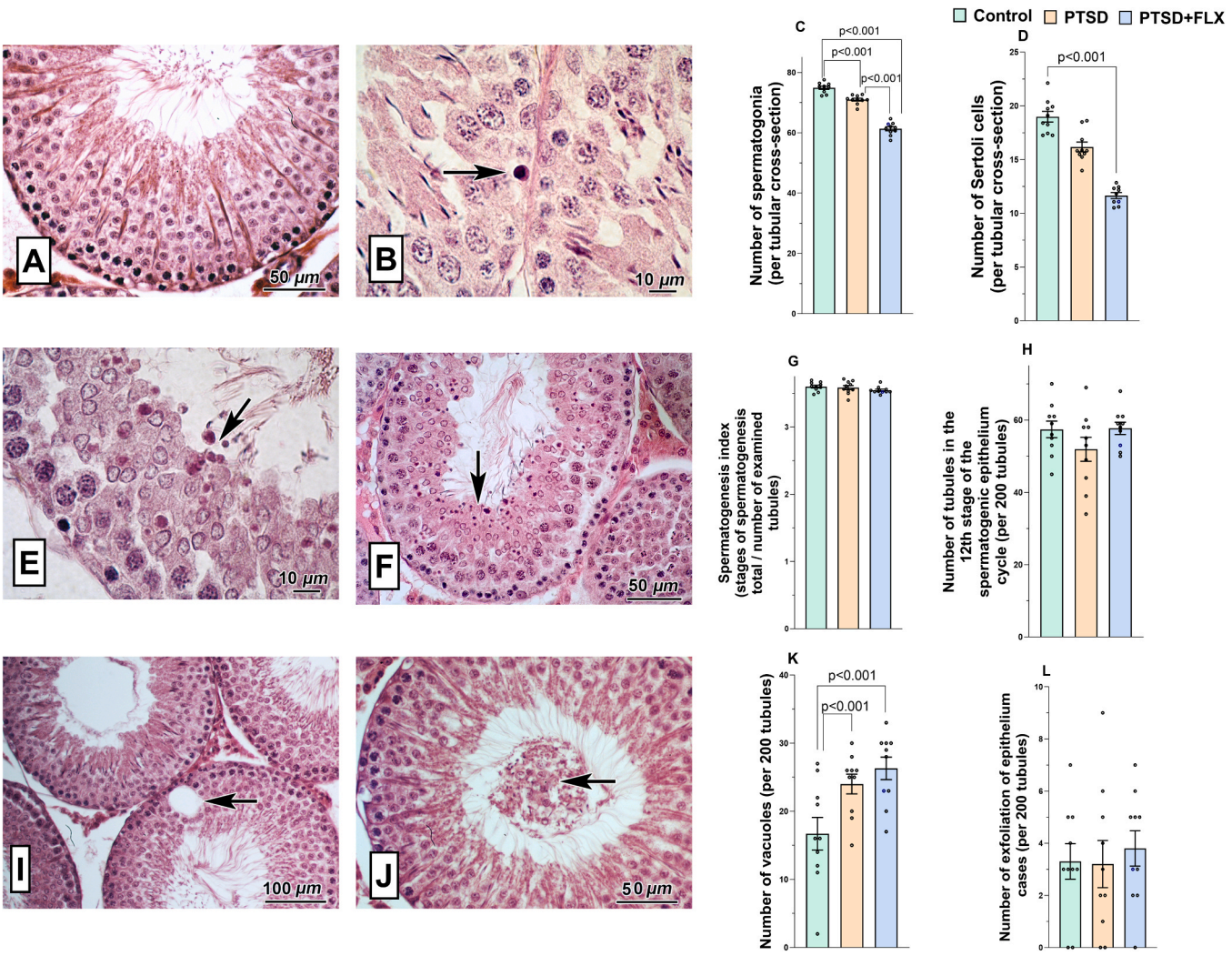

Fig. 6. Histological changes in the testes of PTSD model rats and FLX-treated rats (A - unchanged histological structure of control group rat testes; B - PTSD: apoptotic body above the basal membrane of the seminiferous tubule (arrow); C - quantitative data: number of spermatogonia; D - quantitative data: number of Sertoli cells; E and F - PTSD and PTSD+FLX: degenerative changes in the spermatogenic epithelium in the adluminal compartment of the seminiferous tubules (arrows); G - quantitative data: spermatogenesis index; H - quantitative data: number of tubules in the 12th stage of the spermatogenic epithelium cycle; I - PTSD+FLX: intra-epithelial vacuole in the seminiferous tubule (arrow); J - PTSD+FLX: exfoliated degenerative cells in the lumen of the seminiferous tubule (arrow)). Testis cross-sections: hematoxylin and eosin staining. Quantitative data: the graphs represent means±SEMs. The indicated *p* values were obtained using one-way ANOVA, followed by a post hoc Tukey test.

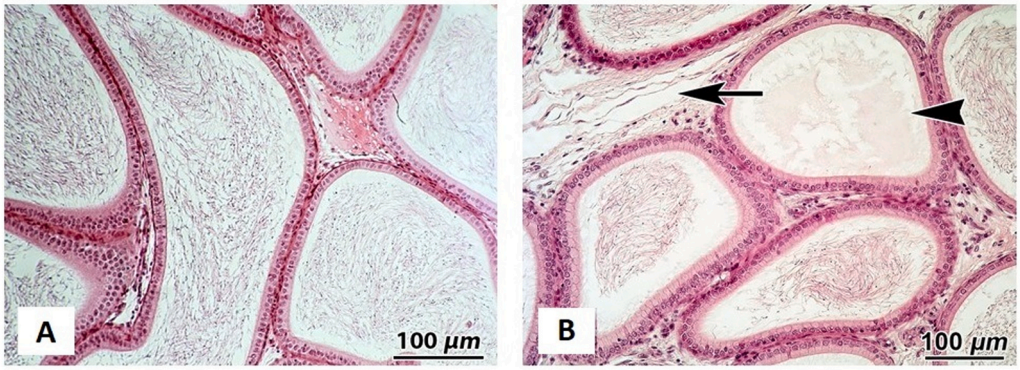

Fig. 7. Histological structure of the epididymis (A - unchanged histological structure of the epididymides of the control group; B - efferent ducts of the typical structure lined with pseudostratified columnar epithelium, spermatozoa and a small amount of light fluid densely filling the lumens (PTSD+FLX)). Hematoxylin and eosin staining.

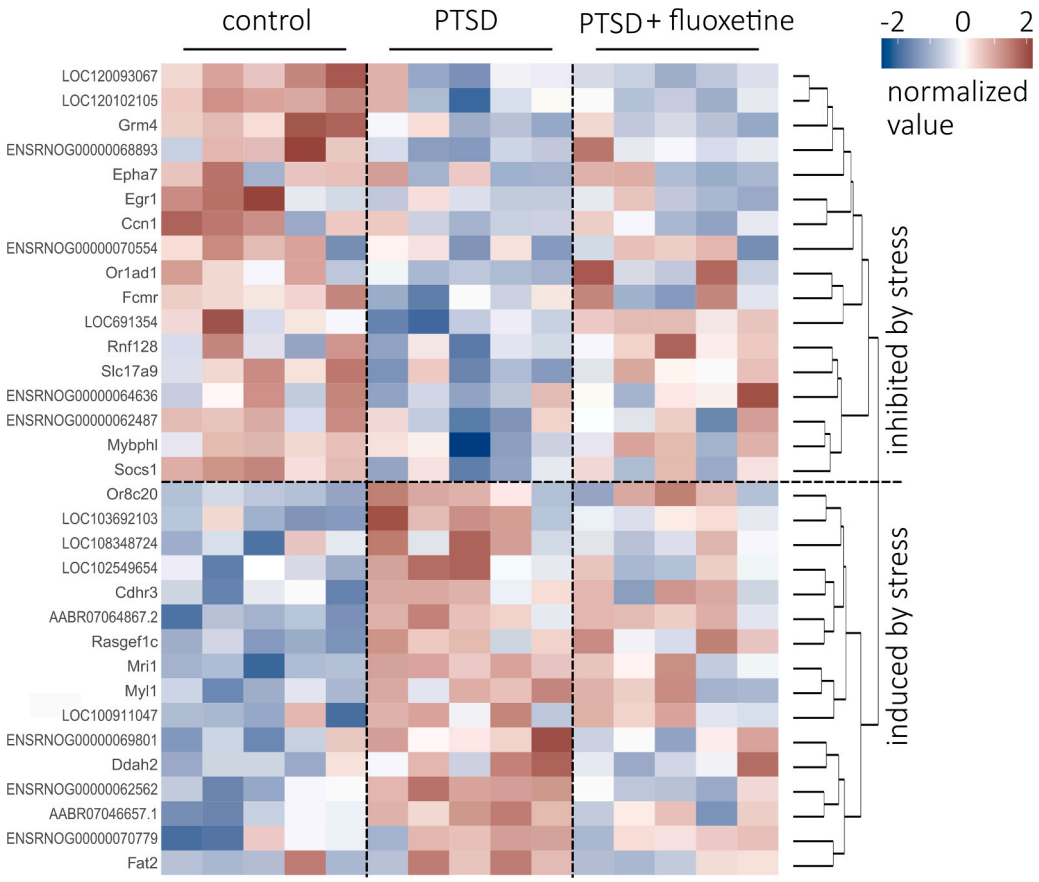

Fig. 8. The heatmap displays relative differences in transcript abundance levels for 33 genes with > 1.5-fold changes (nominal $p < 0.001$ via EdgeR analysis) between the control and stress groups. Each column represents an individual sample, which were grouped as indicated by the labels above the columns. Rows correspond to transcripts labeled with gene symbols (or Ensembl IDs in cases where gene names were unavailable). The transcripts are ordered by hierarchical clustering (dendrogram on the right). The color scale reflects row-normalized z scores (calculated as the deviation from the row mean divided by the standard deviation). Dashed lines were added to delineate the experimental groups and separate transcripts with increased versus decreased abundances after the stress procedure.

experiment. For example, the administration of 20 mg/kg fluoxetine over a short time period (11 days) has been associated with significant reductions in testosterone levels [58]. Moreover, significant decreases in testosterone and FSH levels were observed in rats exposed to fluoxetine at a concentration of 200 mg/kg for 60 days [59]. Similar changes were observed in adult male rats that were orally treated with 10 mg/kg fluoxetine for 8 weeks [60]. Conversely, in another study involving the same treatment duration and drug dose, fluoxetine did not affect these hormones in rats [61]. Furthermore, the administration of fluoxetine (5 mg/kg) to prepubertal rats alters LH, FSH and testosterone levels upon reaching adulthood [15].

The sperm count remained unaffected in rats subjected to PTSD at peripubertal ages, despite the impairment of FSH and testosterone levels. Conversely, in the PTSD + FLX group, reductions in all three of the previously mentioned sperm parameters (including the sperm count) were observed. This discrepancy could be linked to different cellular targets and mechanisms involved in an imbalance of sperm production in untreated and treated animals with PTSD. A reduction in testosterone may not immediately affect sperm production when considering the initial age of the animals included in our study and the duration of the spermatogenic cycle in the rats. The complete process of spermatogenesis in rats spans approximately 50 days, beginning from the initiation of stem cell division to the formation of mature spermatozoa [62]. Given this extended timeline, transient decreases in testosterone may not immediately affect sperm parameters as the existing cohort of developing germ cells continues to mature. Therefore, the PTSD induction in our model may not have been sufficient to elicit a measurable decrease in the sperm count. Moreover, fluoxetine administration during the pubertal period appeared to exacerbate the reproductive disturbances caused by stress, as indicated by a significant reduction in the sperm count in the PTSD + FLX group. This effect was expected and has been reported by numerous researchers [16,59,63–65]. Fluoxetine likely interferes with gonadal function via mechanisms linked with hormonal impairments and alterations of the spermatogenic epithelium (discussed below), as well as via impairments of epididymis contraction and alterations in sperm transit time and count [66]. However, in this group of animals, some degenerative changes in the epididymides cannot be excluded, due to the fact that their weights were significantly reduced. Indeed, the histological examinations revealed moderate dystrophic alterations in the epididymides in the PTSD+fluoxetine group, although all of the lumens of the ejaculatory ducts were populated with spermatozoa. The observed decrease in epididymis weight aligns with the results of other studies, in which rodents were administered different fluoxetine doses in adulthood [59,67] or exposed to the drug via the placenta and mother's milk [68].

Compared with that of the epididymides, the relative weight of the testes (based on the gonadosomatic index) was greater in the PTSD+FLX rats. This finding is inconsistent with the findings of other authors who have reported either no changes or decreases in this parameter following fluoxetine exposure [60,65,69,70]. One well-known cause of an increase in testis weight can involve interstitial edema resulting from alterations in hemodynamics, injury to the vascular endothelium, and reduced lymphatic drainage [71]. Indeed, histological examinations of rat testes from both the PTSD and PTSD+FLX groups revealed pathological

changes indicative of blood circulation disturbances in small-caliber arteries and veins, as well as abnormalities in interstitial capillaries, uneven blood filling of small blood vessels, and venous congestion in both groups with PTSD. Nonetheless, the increase in the gonadosomatic index observed in the fluoxetine-treated group indicated more profound vascular abnormalities. Although direct studies of PTSD or the effects of fluoxetine on the testicular vasculature are limited, we can speculate that the observed phenomenon may be related to a redox imbalance. It has been hypothesized that chronic PTSD can lead to mitochondrial dysfunction and potentiate oxidative stress in the entire organism [72,73]. Other available evidence indicates a significant role of oxidative stress in the mechanisms involved in testicular damage caused by fluoxetine [55,74]. In addition, according to recent data, PTSD is associated with persistent, low-degree inflammation [75]. Both oxidative stress and inflammation can impair endothelial function and disrupt microcirculation [76]. Despite evidence of a beneficial effect of fluoxetine on endothelial function [77], *in vitro* experiments involving carotid arteries from apolipoprotein E-deficient mice have revealed that fluoxetine can augment vascular permeability and increase the chemokine-induced integrin-binding activity of circulating leukocytes [78]. The abovementioned data align with our observation of numerous peritubular edema foci in some areas of the testes in the PTSD and PTSD+FLX groups, which most likely resulted from increased blood vessel wall permeability. The expansion of the peritubular spaces caused disorganization of the collagen fibers in the local connective tissue, in addition to the disarray of compartment-shaped cells and interstitial endocrinocytes. These cells experienced dystrophic and destructive changes, including pericellular swelling. The disturbance of Leydig cell integrity, as well as the decrease in the number of Leydig cells observed in our study, were likely the main causes of suppressed testosterone production and the decrease in serum testosterone levels. Correspondingly, the observed endocrinocyte impairment and testosterone deficiency may have been responsible for changes in sustentocytes [79].

Hormonal abnormalities and disturbances in the testicular blood circulation system, as well as the appearance of edematous changes in the peritubular connective tissue, often lead to the development of dystrophic/destructive changes in spermatogenic epithelium cells [71]. In our study, these changes included a significantly greater number of tubules with disarranged epithelium and signs of increased apoptosis. The latter effect was reflected by a more frequent occurrence of dense apoptotic bodies among cells located on the basal membrane of the seminiferous tubules (presumably involving sustentocytes and spermatogonia). Notably, the rats used in this experiment were young, and we cannot neglect the fact that increased numbers of degenerating (apoptotic) cells are also associated with immaturity [71]. However, quantitative analyses revealed a reduction in the number of spermatogonia in both PTSD groups, with a more pronounced decrease being observed following fluoxetine treatment. In addition, a significant reduction in the number of Sertoli cells in the PTSD+FLX group was demonstrated. This finding aligns with previous studies indicating that fluoxetine exposure can decrease Sertoli cell populations [80].

This effect of fluoxetine on Sertoli cells during the pubertal period could lead to many destructive changes in the seminiferous epithelium. The Sertoli cell has several important roles in spermatogenesis, including support and nutrition of the developing germ cells; compartmentalization of the seminiferous tubule by tight junctions; controlled release of mature spermatids into the tubular lumen (spermiation); secretion of fluid, proteins and several growth factors; and phagocytosis of both the degenerating germ cells and the excess cytoplasm (residual body) that remains from released sperm [81]. The cytoskeleton of terminally differentiated mammalian Sertoli cells represents one of the most elaborate cytoskeletons of those described for cells in various tissues. Actin filaments, intermediate filaments and microtubules demonstrate distinct patterns of distribution that change during the cyclic process of spermatogenesis [81]. The microtubule cytoskeleton of sustentocytes defines elongated spermatid locations within tubules, and failure of the cytoskeleton causes abnormal detachment and apoptosis of these cells [81,82]. We noted an increase in the number of cases of detachment of the seminiferous epithelium from the basement membrane and sloughing into the seminiferous tubule lumen in the PTSD+FLX group, and this observation was confirmed by quantitative data. This phenomenon suggests potential effects on the Sertoli cell cytoskeleton, as such events are associated with Sertoli cell microtubule destabilization [83].

In addition to the detachment of the seminiferous epithelium, the frequencies of intraepithelial vacuoles in seminiferous tubule basal sections were increased in the untreated and treated PTSD groups. Seminiferous epithelium vacuolization is a relatively common histopathological observation associated with Sertoli cell injury [83].

The Sertoli cell also mediates the actions of FSH and LH-stimulated testosterone production in the testis, which apparently occur in a stage-dependent manner [81]. Moreover, FSH regulates Sertoli cell proliferation; thus, a decrease in FSH levels could be one of the reasons explaining the decline in Sertoli cells in the PTSD+FLX group [84].

Taken together, our histological findings indicate that PTSD simulation and chronic fluoxetine administration lead to impaired spermatogenesis, which is due to degenerative processes affecting both germ cells, sustenocytes, and endocrinocytes; these processes mainly occurred in the drug-treated group. These changes may result from a direct effect of fluoxetine on various cell types within the seminiferous epithelium or from a secondary effect of fluctuations in hormone levels, modifications in blood supply, or disturbances in fluid dynamics, regardless of whether these changes occur in the testis or the epididymis.

Despite altered morphological features, gene expression analysis revealed relatively mild stress-induced changes and no fluoxetine-induced changes in gene expression in the testes. This result does not exclude a pronounced role of gene regulation in the establishment of the observed disturbances. Relevant but transient gene expression changes may have occurred and subsequently subsided before the start of the analysis, which was performed 28 days after the stress procedure and 7 days after fluoxetine treatment was completed. Changes in gene expression observed due to the less stringent significance criteria could be considered to be consistent with the deleterious effects of stress observed using other methods. For example, the downregulation of the immediate-early gene *Egr1* in the testes could hamper spermatogenesis, due to the fact that the Egr1 protein directly and indirectly regulates the expression of genes critical for cell proliferation [85]. Moreover, *Egr1* (along with *Sp1* and *Sp3*) plays a critical role in the regulation of *Dmrt1*, which is expressed in Sertoli cells and is required for postnatal testis differentiation [86]. Thus, *Egr* downregulation may contribute to the histological abnormalities observed in the testes of rats experiencing stress at peripubertal ages. In contrast, the increased abundance of the transcript encoding myosin light chain 1 (*Myl1*) in the testes of stressed rats may represent a compensatory reaction. As a part of the myosin network [87], *Myl1* can play a role in intracellular transport, post-Golgi transportation, and acrosome biogenesis [88]. During spermiogenesis, myosins are involved in acrosome formation, nucleus morphogenesis, mitochondrial translocation, and the individualization of spermatids [88]. Therefore, upregulated *Myl1* can contribute to the restoration of spermatogenesis disturbed by stress. Finally, an unexpected observation was the stress-induced decrease in transcription of the metabotropic glutamate receptor 4 gene (*Grm4*). This gene is highly expressed in the central nervous system, where it is involved in the control of excitatory nerve transmission [89]. Furthermore, it is also expressed in spermatogonia and late spermatids [90]; thus, its potential involvement in the long-term effects of stress in the testes merits further investigation.

One limitation of the present study is the absence of a fluoxetine-only group. While this may somewhat limit the ability to fully disentangle the isolated effects of fluoxetine from those of PTSD, the primary aim of this study was to evaluate their interaction during a critical developmental window. Moreover, the effects of fluoxetine alone have been extensively characterized in previously published studies cited in this manuscript,

which provides a framework for interpreting our findings.

5. Conclusion

This study provides novel translational insights into the long-term consequences of fluoxetine administration on the male reproductive system in the context of PTSD in peripubertal individuals. Our findings demonstrate that exposure to strong stressors alone disrupts the HPG axis, thereby leading to reductions in FSH and testosterone levels, whereas fluoxetine administration does not ameliorate these alterations; rather, it contributes to additional reproductive impairments. Histological analyses revealed structural alterations in the testes and epididymides, including vascular abnormalities, interstitial edema, and dystrophic changes in the seminiferous epithelium. Thus, fluoxetine exposure occurring in PTSD during puberty may contribute to testicular dysfunction both through hormonal dysregulation and by impairing local testicular microcirculation. The loss of Sertoli cells was particularly evident in the fluoxetine-treated group; moreover, given their essential role in maintaining spermatogenesis, this impairment may underlie the observed reductions in sperm count and the overall disruption of the spermatogenic process.

These results highlight the detrimental impact of fluoxetine on male reproductive function, particularly in the context of PTSD-induced hormonal dysregulation. Although fluoxetine is widely used for treating PTSD and other psychiatric conditions, its influence on reproductive health underscores the need for a cautious approach when prescribing this drug to males (particularly to adolescents, in whom pubertal hormonal maturation is still ongoing).

CRediT authorship contribution statement

Alla Voronina: Investigation, Formal analysis. **Jan Rodriguez Parkitna:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis. **Serhii Luhovskyi:** Writing – original draft, Investigation. **Michał Korostyński:** Writing – review & editing, Formal analysis. **Ganna Shayakhmetova:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Jacek Hajto:** Investigation, Formal analysis. **Maksim Munko:** Investigation. **Larysa Bondarenko:** Investigation, Formal analysis. **Tetiana Karatsuba:** Investigation. **Barbara Ziółkowska:** Writing – review & editing, Writing – original draft. **Sławomir Gołda:** Investigation. **Valentina Kovalenko:** Investigation. **Iryna Blazhchuk:** Investigation.

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.

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