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Valproic acid during pregnancy decrease the number of spermatogenic cells and testicular volume in the offspring of mice: Stereological quantification

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Summary. Valproic acid (VPA) is a drug used to treat epilepsy, bipolar disorders and headaches. As a secondary effect, this antiepileptic drug can cause a decrease in androgens and gonadotropins, and dose-dependent testicular defects, such as reduction of testicular weights, sperm motility and degeneration of the seminiferous tubules. In offspring exposed to VPA, its effects have not been evaluated, so the study aimed to determine the morphological effects of the use of VPA along testicular development in mice. 30 adult female BALB/c mice were crossed and divided by age, with embryos of 12.5 days post coitum (dpc), fetuses of 17.5 dpc and male mice 6 weeks postnatal. In each case, the pregnant mouse received 600 mg/kg of VPA, making up the VPA groups, or 0.3 mL of 0.9% physiological solution for the control groups, from the beginning to the end of the pregnancy, orally.t. A morpho-quantitative analysis was carried out on the gonadal development of the male offspring. In the groups treated with VPA, at all ages studied they had lower testicular volume. At 12.5 dpc, they showed less testicular development in the form of sex cords, with fewer gonocytes and somatic cells. At 17.5 dpc, they presented greater interstitial space, fewer spermatogonial, sustentacular Sertoli, peritubular and interstitial Leydig cells. At 6 weeks postnatal, they presented fewer spermatogonia, pachytene spermatocytes, elongated spermatids, sustentacular Sertoli and interstitial Leydig cells, with statistically significant differences. In conclusion, prenatal exposure to VPA causes histopathological alterations in the offspring of mice in testicular development, from the embryonic stage to 6 weeks postnatal.

Key words: Valproic acid, Testicular development, Spermatogenesis, Alterations, Congenital malformations

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Introduction

Epilepsy is a neurological disorder that affects 50 million people worldwide, and is considered the most prevalent chronic non-communicable neurological disease. Of these patients, about 80% belong to low- and middle-income countries. It is estimated that around 2.4 million people are diagnosed with epilepsy in the world every year (World Health Organization, 2019). In turn, it is one of the most common chronic diseases in women of reproductive age, having the possibility of pregnancy during this period (Viale et al., 2015).

Among the first-line drugs for the treatment of epilepsy are carbamazepine, phenytoin and valproic acid (VPA), using the most appropriate drug as monotherapy at the minimum dose capable of controlling the disease (Manford, 2017). Of these, VPA is one of the most

Abbreviations. VPA, Valproic acid; GABA, Gamma aminobutyric acid; PGCs, Primordial germ cells; HDAC, Histone deacetylase; TGF-β1, Transforming growth factor β1; dpc, Days post coitum; mg, Milligram; kg, Kilogram; mL, Milliliter; NaCl, Sodium chloride; L, Liter; M, Molar; μm, Micrometer; H&E, Hematoxylin and eosin; t, Thickness; A, Total area; $V_{(total)}$, Total volume; d, Distance; P, Points; N, Number of cells; Q, Particles; DAB, Diaminobenzidine chromogen; 8-oxo-dG, 8-Oxo-2'-deoxyguanosine; ROS, Reactive oxygen species; DNA, Deoxyribonucleic acid.

prescribed due to its great efficacy, which is explained by an increase in the levels of gamma aminobutyric acid (GABA) in the brain, which, if found at insufficient levels, produces seizures (Morland et al., 2012), as well as inhibiting voltage-dependent sodium channels, neuronal metabolism, and histone deacetylases (HDAC) (Ornoy, 2009; Ghodke-Puranik et al., 2013). That is why it is also used as a treatment for mood disorders, bipolar disorders, and headaches (Khan et al., 2016).

During pregnancy, the use of antiepileptic drugs carries a significant risk for the development of the embryo and fetus, since a cause-effect relationship has been demonstrated between the use of antiepileptic drugs and the incidence of congenital malformations, increasing the risk of acquiring them between 5 to 9% (Ahmed et al., 2014). Among the first-line drugs, VPA is estimated to be the most teratogenic, with a risk of causing malformations 3 times higher compared to carbamazepine and phenytoin (Ornoy, 2009). It is even recommended to avoid the use of this drug when the patient's conditions allow it for another with a lower risk of malformation. However, VPA intake during pregnancy is essential for women with some generalized epilepsy syndromes, such as myoclonic epilepsy, and it has better documented efficacy than alternative drugs. Thus, it is advisable not to discontinue their use and to have adequate medical advice (Tomson et al., 2016; Macfarlane and Greenhalgh, 2018).

In pregnancy, VPA can produce possible adverse effects on the development of various systems of the embryo and fetus, such as gastrointestinal, neurological, hematological and reproductive (Tanoshima et al., 2015). Also, clinical studies of children suggest that exposure to VPA in utero may cause fetal valproic acid syndrome, which has characteristics similar to autism spectrum disorder (Christensen et al., 2013). Such prenatal and postnatal defects have been found in rodents exposed prenatally to VPA (Schneider et al., 2006; Roullet et al., 2010).

Throughout the development of the male reproductive system, despite the antioxidant protection present to support its dual functions of steroidogenesis and spermatogenesis, a wide variety of endogenous and exogenous factors are known to disrupt these defenses and generate a state of oxidative stress (Aprioku, 2013). In this sense, it has been seen that VPA is associated with endocrine disorders, decreasing serum concentrations of androgens and gonadotropins in men with epilepsy (Rättyä et al., 2001). Also, infertility has been reported in male subjects taking VPA for epilepsy (Yerby and McCoy, 1999). An *in vitro* study determined that VPA may have more negative effects than other antiepileptic drugs such as carbamazepine, phenobarbital or phenytoin, on sperm motility (Chen et al., 1992).

In animal models, VPA has been shown to cause dose-dependent testicular defects, observing a decrease in testicular, epididymal, seminal vesicle and prostate weights, a reduction in the number of sperm heads in the caudal epididymis and the percentage of sperm motility, as well as degeneration of the seminiferous tubules and the loss or exfoliation of the spermatids at higher doses (Nishimura et al., 2000; Sveberg Røste et al., 2001). Other investigators further demonstrated that apoptotic cell counts and p53 immunoreaction were high and TGF-β1 expression was lower (Cansu et al., 2011). The latter showed that treatment with VPA from prepuberty to adulthood negatively affects spermatogenesis.

To date, the effects on testicular development in offspring of mice exposed to VPA have not been evaluated. We hypothesized that the prenatal administration of VPA generates morphological alterations throughout the gonadal development of the litters, in the embryonic, fetal and postnatal stages. For this reason, this study was designed to determine the morphological effects of the use of VPA at the testicular level in embryos and fetuses of BALB/c mice of 12.5 and 17.5 days post coitum (dpc), as well as at 6 weeks postnatal.

Materials and methods

Formation of study groups

The study was approved by the Scientific Ethics Committee of Universidad de La Frontera, Temuco, Chile, act nº 122_18 in December 2018. Thirty pregnant adult female mice (*Mus musculus*) BALB/c from the Bioterium of the Doctoral Program in Morphological Sciences of Universidad de La Frontera were used. These remained during the experimental protocol under 22±2°C with 50-70% humidity and a light/dark cycle of 12 h (08:00-20:00/20:00-08:00), being maintained with a standard laboratory diet (AIN-93M) (Reeves et al., 1993) and water *ad libitum* for their adaptation to the new environment.

Subsequently, the pregnant mice were randomly divided into 6 groups of 5 mice each. The moment when the mucous plug was observed at the vaginal level (which means that there was copulation) was considered as 0.5 dpc.

According to age, they were divided into 12.5 dpc embryos, 17.5 dpc fetuses, and male mice 6 weeks postnatal, forming two groups for each one. In these stages, the beginning of the formation of the testicular sex cords can be observed at 12.5 dpc. After 17.5 dpc, they are already formed and the stereodogenic function begins in the interstitial cells, marking the end of prenatal development, observing the gonad moments before delivery (Yildirim et al., 2020). In addition, in mice, parturition can occur from 18 dpc and infanticide generally occurs in offspring with defects (Kuroda and Tsuneoka, 2013). At 6 weeks postnatal, the pubertal period begins in this model, which is correlated with a peak in body and testicular weight gain, greater quantity, volume and area of seminiferous tubules and complete development of the germinative epithelium, compared to previous or later stages, due to the activation of the hypothalamic-pituitary-testis axis

(Montoto et al., 2012). The VPA groups received 600 mg/kg of VPA (Atemperator®, Recalcine® Laboratory) and the others were administered 0.3 mL of 0.9% physiological solution (0.9% NaCl), making up the control groups, according to a previous model (Conei et al., 2016). At these doses it has been seen to cause skeletal, cardiovascular, urogenital and neural tube defects, without affecting the size and viability of the litter (Binkerd et al., 1988; Bass et al., 2020). All groups received their respective doses orally by gavage from the moment the mucous plug was visualized in the vaginal introitus (time 0.5 post-fertilization) until the end of pregnancy and their body weight was measured at the beginning, during (once a week) and at the end of the experiment with an IKA C–MAGHS7 balance. Finally, the sample size of male offspring was made up of: 12.5 dpc control group 20 (4±1.41 male embryos per pregnancy) and VPA group 25 (5±2.00 male embryos per pregnancy); 17.5 dpc control group 21 (4.2±1.30 male fetuses per pregnancy) and VPA group 23 (4.6±1.14 male fetuses per pregnant pregnancy); 6 weeks postnatal control group 29 (5.8±1.30 pubertal male per pregnancy) and VPA group 24 (4.8±1.30 pubertal male per pregnant pregnancy) (Fig. 1).

Euthanasia

Experimental protocol included the administration of a combination of dissociative anesthesia with xylazine at a dose of 10 mg/kg and ketamine at a dose of 80 mg/kg intraperitoneally 3 times. For euthanasia, cervical dislocation was performed (American Veterinary Medical Association, 2013). The uterus was dissected and embryos were removed. They were deposited in saline, quantifying their number and obtaining their apex-caudal length. The uterus was visualized to find possible areas of abortion. This was done at 12.5 and 17.5 dpc. Once the fetuses were extracted, they were euthanized through decapitation with a scalpel (American Veterinary Medical Association, 2013). Meanwhile, at 6 weeks postnatal, the same doses were used and the testes were extracted, which were measured in length, width and depth.

Morpho-quantitative analysis

Processing and staining

Embryos, fetuses and pubertal testes were fixed in buffered formalin (1.27mol/L formaldehyde in 0.1 M phosphate buffer pH 7.2) at 10% for 48 h, dehydrated, embedded in Paraplast Plus and (Sigma-Aldrich Co., St. Louis, MO, USA). Once the blocks were obtained, serial cuts of 10 μm thickness were made in a microtome (Microm HM 325, Thermo Scientific™ , MA, USA) in a longitudinal serial manner for each block of embryos and fetuses, and serial cross-sections for pubertal mice.

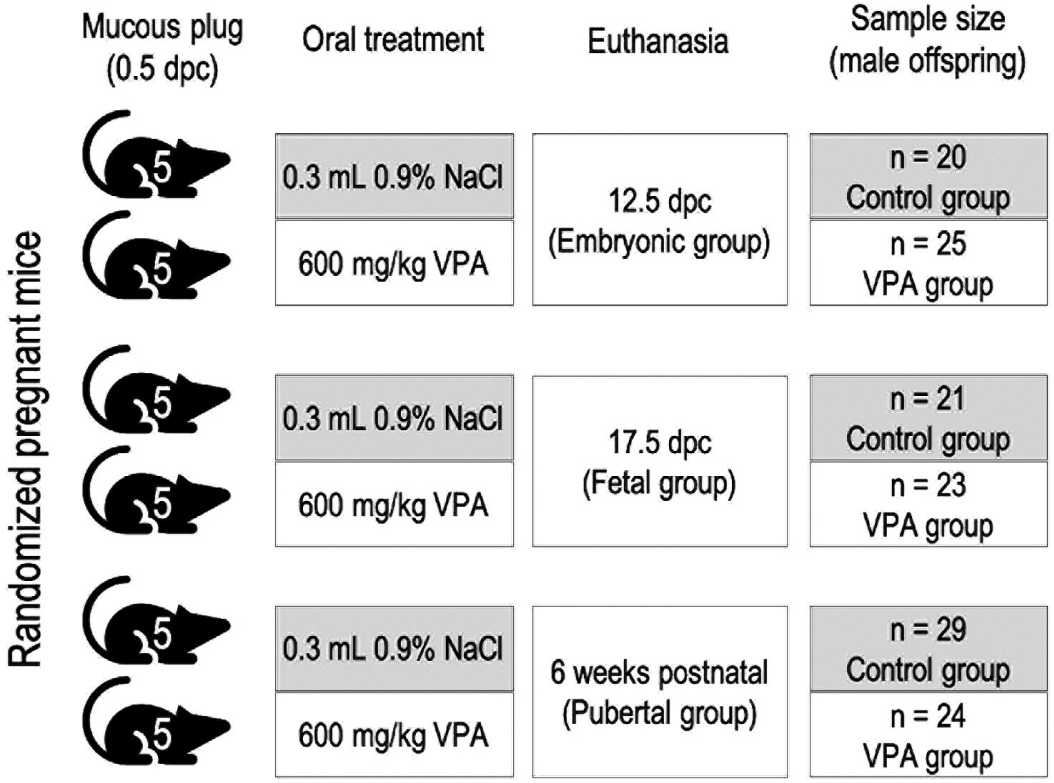

Fig. 1. Experimental design. 5 pregnant BALB/c mice were used per group. Days post-coitum (dpc). Valproic acid (VPA). Sample size (n).

Histology

Sections were stained with hematoxylin and eosin (H&E). The slides were viewed under an Olympus microscope equipped with a motorized stage controlled by Cast-Grid stereological software (Stereology Software Package, Silkeborg, Denmark, https://visiopharm.com/) and images were captured with a DP 70 color digital camera 12.5 megapixels (Olympus Corporation of the Americas, PA, USA). This camera employs a single-chip charge-coupled device (CCD) sensor with Bayer RGB primary color filtration, and produces 36-bit RGB color depth for recording subtle color intensities and gradations. The XY displacement of the microscope stage was managed and allowed the selection of visual fields by systematic random sampling after the entry of an adequate sampling fraction (Gómez et al., 2009). A morphological description of the cells present in the tubular, peritubular and interstitial compartments was made in terms of the morphology of the germinative epithelial, sustentacular (Sertoli), peritubular and interstitial (Leydig) cells, in addition to their location and organization in each of the samples analyzed.

Johnsen score

To complement the histopathological analysis, the histological quantification was performed in the testicular sections assigning a Johnsen score according to the following criteria (Glander et al., 2000; Khan et al., 2011; Rafiee et al., 2019):

Score 1: Absence of cells in tubular section.
Score 2: Absence of germ cells.
Score 3: Presence only of spermatogonia.
Score 4: Presence of few spermatocytes (<5).
Score 5: Absence of spermatids.
Score 6: Presence of a few early spermatids (<5-10) and no spermatozoa.
Score 7: Absence of late spermatids.
Score 8: Presence of a few late spermatids (<5-10).
Score 9: Disorganized tubular epithelium.
Score 10: Complete spermatogenesis.

For this, 15 sex cords were randomly examined for the 12.5 and 17.5 dpc groups and 30 seminiferous tubules in the case of pubertal mice. Johnsen's score was calculated by dividing the sum of all scores by the total number of sex cords or seminiferous or tubules examined.

Stereology

From the serial cross-sections obtained, the testicular volumes were determined using the Cavalieri principle (Gundersen and Jensen, 1987; Altunkaynak et al., 2009). According to the Cavalieri principle, the volume of an object can be estimated by multiplying the surface area of the biological object of interest defined by a specific point count grid by the thickness of each histological section. For this, the initial section to be analyzed was randomly obtained from the first six sections, to later analyze the next histological sample every five histological sections. The point densities of the 100-point counting grid were designed based on previous studies, being sufficient to obtain a significant error coefficient (Gundersen, 1986; Dursun et al., 2010; Altunkaynak et al., 2015). The images were processed with the STEPanizer (https://www.stepanizer.com/), a computer-based software tool, programmed through JAVA® (Oracle Corporation, Redwood Shores, CA, USA) for the stereological evaluation of digitally captured microscopic images of all kinds, providing a defined workflow through the use of basic stereological tools, such as overlay test systems, a scaling function, a counting module, and an export function for transferring results to spreadsheet programs (Tschanz et al., 2011). For stereological processing, a 100-point lattices test system was superimposed onto the images. The areas of the testes were calculated. The value of the measured surface area was multiplied by the thickness of the section (t) and volumetric results were obtained, using the following formula (Altunkaynak et al., 2015): $V_{(total)} = t \cdot \Sigma A$, where "t" is the thickness of the section (including intervals) and ΣA is the total area of the testis specimen section. The term ΣA is equal to: $\Sigma A = d^2 \cdot \Sigma P$, where d^2 is the distance between points and ΣP is the number of points that contact favorable areas in the sections.

The total number of gonocytes ($N_{Gonocyte}$), somatic cells undifferentiated in interstitial space ($N_{Somatic}$), spermatogonias ($N_{Spermatogonia}$), pachytene spermatocytes ($N_{P\text{-}Spermatocyte}$), round spermatids ($N_{R\text{-}Spermatid}$), elongated spermatids ($N_{E\text{-}Spermatid}$), sustentacular Sertoli ($N_{Sertoli}$), interstitial Leydig (N_{Leydig}) and peritubular cells ($N_{Peritubular}$) was estimated through the optical disector method, where only the nuclei that meet the inclusion criteria defined by Sterio's rule were considered for the counting (Weibel, 1970; Gundersen and Jensen 1987). In serial sections, the first of the series selected randomly from the first six sections was analyzed, and every five sections the successive section of the series was compiled. The total number of cells was calculated from the number of cells counted and the probability of sampling (Weibel, 1970; Gundersen, 1986). The total number of testicular cells was calculated by:

$$N_V = \frac{\sum Q}{a \times h}$$

where N_V is the numerical density of testicular cells, ΣQ total number of particles in the disector, h=5 μm (the height of the disector) is the distance between the two selected optical planes in the thickness of the physical cut to delimit the volume of the disector, and a is the area of the disector. The total number of cells (N) was

then equal to N_V multiplied by the total testicular volume.

Statistical analysis

Quantitative data were expressed as means ± standard deviation and were evaluated using the Kolmogorov-Smirnov test (data normality analysis). To determine differences in the means of Johnsen's score, morphometric and stereological variables, depending on the normality of the data, they were analyzed using Student's t-test or Mann Whitney's U, as appropriate. A value of $p \leq 0.05$ was considered statistically significant (GraphPad Prism Software Inc., 8.0, CA, USA).

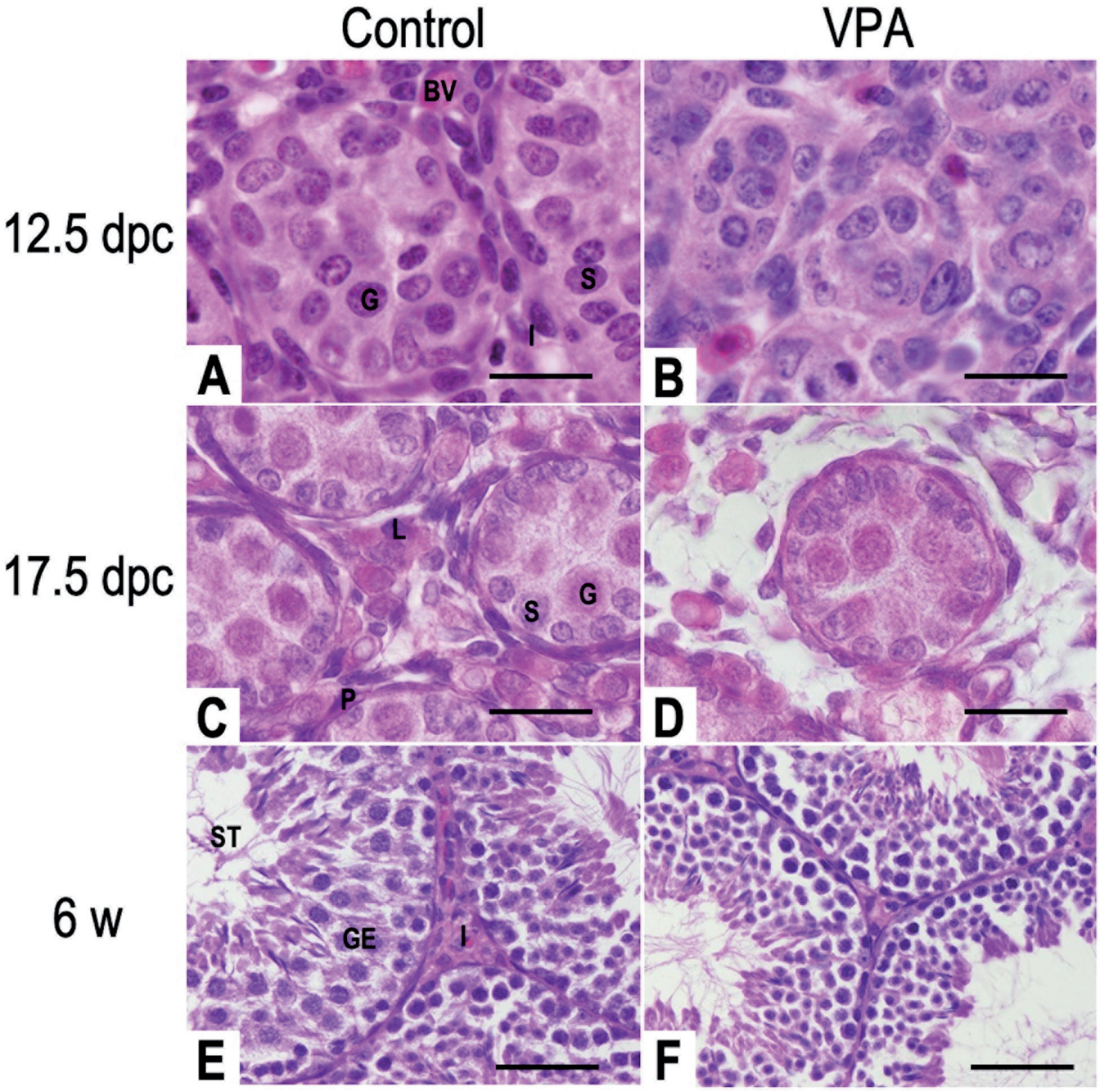

Fig. 2. Comparison between control groups and those treated with valproic acid (VPA). In **A** and **B** groups of 12.5 days post-coitum (dpc). In **C** and **D** groups of 17.5 dpc. Bar 20 μm. In **E** and **F** groups of 6 weeks (w). Bar 50 μm. (G) Gonocyte, (S) Sustentacular Sertoli cell, (I) Interstitial tissue, (BV) Blood vessels, (P) peritubular cell, (ST) Seminiferous tubules, (GE) Germinal epithelium.

Results

Histology

At 12.5 dpc, in the VPA group, the testis is recognized by the presence of tunica albuginea and sex cords, without significant differences compared to the control, but the peritubule has not been completely differentiated in interstitial tissue. Pre-sustentacular Sertoli and pre-spermatogonia cells are seen in the cords. Cells that do not constitute cords correspond to the interstitium and have capillaries and interstitial Leydig cells. The sex cords do not have a defined shape and the testicular parenchyma is not defined, compared to the control group (Fig. 2A,B).

At 17.5 dpc, in the fetal stage, in control and VPA groups the presence of sex cords formed by a layer of peritubular cells is observed, and it is possible to recognize the presence of pre-spermatogonia and differentiated sustentacular Sertoli cells, without an internal lumen. A wide interstitial space is observed in the samples studied (Fig. 2C,D). At 6 weeks postnatal, control and VPA groups demonstrated significant tissue contraction due to paraffin embedding processing (Figs, 2E,F, 3A,B).

Johnsen score

In all the groups analyzed, regardless of age, there were statistically significant differences between the control and VPA groups. At 12.5 dpc, the Johnsen score was higher in the control group with an average of 2.650±0.479 compared to the VPA group with 1.450±0.501 (p-value <0.0001). At 17.5 dpc in the control group, it was 2.790±0.409 and in those treated with VPA it was 2.460±0.502 (p-value =0.0018). At 6 weeks postnatal, the control group obtained a score of 8.970±0.846 and the VPA group of 7.200±1.064 (p-value <0.0001) (Fig. 4).

Morphometry and Stereology

From 12.5 dpc, a smaller testicular volume is already beginning to be evidenced compared to the control group, which is maintained in a sustained way until 6 weeks postnatal, with statistically significant differences (Table 1).

At 12.5 dpc, the control gonad presented a numerical density of gonocytes and somatic cells higher compared to the VPA group. At 17.5 dpc, the development of the different cell types at the testicular level also showed statistically significant differences, being less in the group treated with VPA. This decrease is maintained in pubertal mice, where spermatogonia, pachytene spermatocytes, elongated spermatids, sustentacular Sertoli and interstitial Leydig cells, without finding statistically significant differences in the number of round spermatids and myoid cells (Table 1).

Discussion

In the present study, we detected long-term consequences in the postnatal period, where a decrease in testicular volume and the number of spermatogenic cells were observed after prenatal administration of valproic acid. According to our knowledge, this is the first study that shows long term pubertal consequences of maternal administration of valproic acid during gestation in offspring pubertal testis. This agrees with what was stated by Cansu et al. in 2011, wherein Wistar rats treated with VPA had lower testicular size and weights, as well as the number of spermatogonia and spermatocytes. However, it contrasts with another study in rats that received VPA orally at doses of 250, 500, or

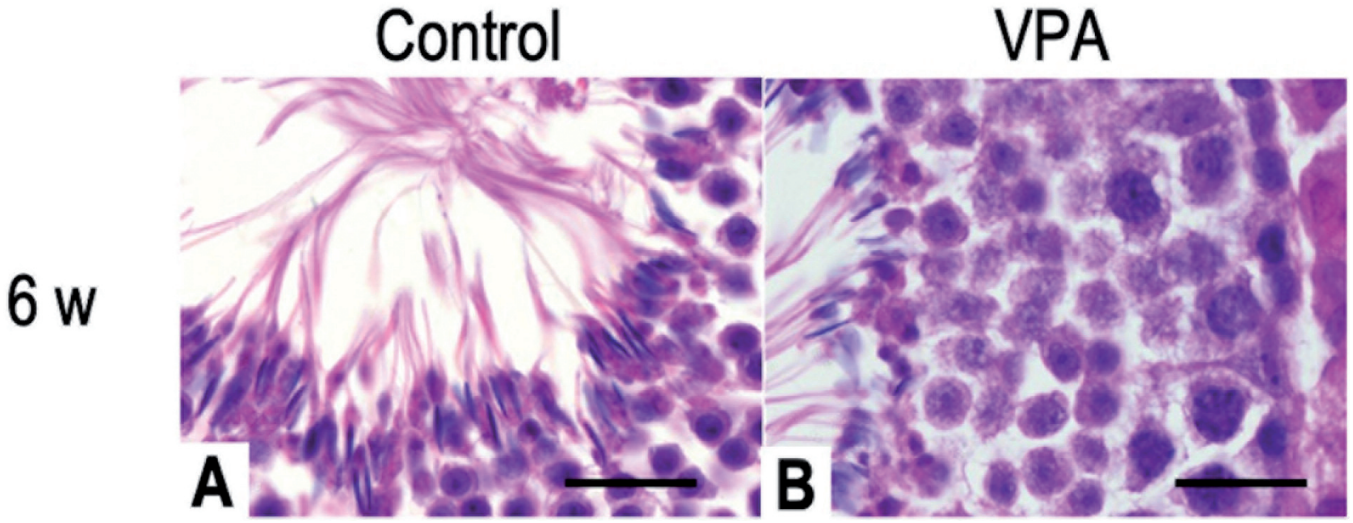

Fig. 3. Comparison between groups of 6 weeks (w). In **A** control group and **B** VPA group. Bar 20 μm.

1000 mg/kg/day for 4, 7, or 10 weeks (Nishimura et al., 2000). In this case, the male rats in the 1000 mg/kg dose group died or were moribund after 3 to 4 days after the start of treatment. In the group that received 500 mg/kg/day of VPA, histopathological examination revealed degeneration of the seminiferous tubules and the loss or exfoliation of the spermatids. This was not present in any group in our study. A similar situation was evidenced by Sveberg Røste et al. in 2001, where they evaluated the morphological changes that VPA can induce at the testicular level in Wistar rats, determining a decrease in testicular weight and generalized testicular atrophy with spermatogenic arrest in treated animals, having greater effects proportionally to the increase in the dose of VPA. Another study determined that the rats treated with VPA presented a small testicular size and in the histopathological analysis an atrophic tunica albuginea, with a reduction in the height of the epithelial layer and wide interstitial spaces measured through morphometric and stereological analyses (Sukhorum and Iamsaard, 2017). The latter is similar to what was stated in our research in the 17.5 dpc group treated with VPA, not at 6 weeks postnatal, where the myoid cells did not present significant differences compared to the control group, which would explain the entire conformation of the peritubular compartment at this age. Another antiepileptic drug such as carbamazepine has shown effects on testicular development and the spermatogenic process at puberty. In rats, it was observed that testosterone levels are decreased, as well as a lower testicular volume and testicular cell count, measured through stereological analyses (de Oliva et al., 2020).

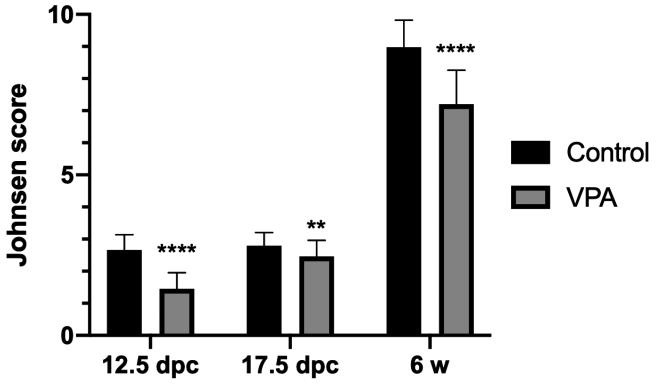

Fig. 4. Comparative graph between control and valproic acid (VPA) groups according to Johnsen's score. 12.5 dpc (post-coital days), 17.5 dpc and 6 w (post-natal weeks). **** p<0.0001, ** p=0.0018.

Prenatal use of VPA has previously been associated with an increased risk of hypospadias (Veroniki et al., 2017). In organotypic culture system of human fetal testes explants with tissue between 10 to 12 weeks of gestation, it was determined that VPA has antiandrogenic properties, decreasing the production of testosterone, without causing tissue toxicity evaluated histopathologically (Gaudriault et al., 2017). Among the drugs linked to alterations in testicular development is metformin, where it has been seen that its prenatal administration in a model similar to the one presented, affects steroidogenesis and decreases testicular volume, the diameter of developing seminiferous tubules and count of sustentacular Sertoli cells, without affecting the apoptotic count (Tartarin et al., 2012).

The most obvious histopathological results were found in the embryonic and fetal groups exposed to the drug. Given this, it could be hypothesized that due to the filter generated by the blood-placental barrier during pregnancy, it is permeable to the diffusion of VPA from the extrauterine to the intrauterine. In this sense, it has been demonstrated during gestation given the lipophilic nature of VPA, postulating a passive diffusion through the placenta (Bailay and Briggs, 2005; Ornoy, 2009;

Table 1. Morphometric and stereological parameters in testicular development of mice treated with valproic acid (VPA) and control (mean±DS).

Stage	Value	Control	VPA	p-value
12.5 dpc	Testicular volume (mm^3)	1.493±0.175	1.384±0.164	0.0162
	$N_{Gonocyte}$	2.402±0.627 x 10^5	2.162±0.623 x 10^5	0.0170
	$N_{Somatic}$	5.806±1.341 x 10^5	4.975±1.392 x 10^5	<0.0001
17.5 dpc	Testicular volume (mm^3)	7.711±0.531	7.328±0.562	0.0141
	$N_{Gonocytes}$	2.711±0.599 x 10^5	1.702±0.402 x 10^5	<0.0001
	$N_{Sertoli}$	5.873±0.993 x 10^5	4.177±0.905 x 10^5	<0.0001
	$N_{Peritubular}$	5.820±0.612 x 10^5	4.017±0.903 x 10^5	<0.0001
	N_{Leydig}	2.100±0.490 x 10^5	1.242±0.358 x 10^5	<0.0001
6 weeks postnatal	Testicular volume (mm^3)	1.558±0.149 x 10^6	1.354±0.162 x 10^6	<0.0001
	$N_{Spermatogonia}$	4.101±0.684 x 10^6	2.900±0.611 x 10^6	<0.0001
	$N_{P\text{-}Spermatocyte}$	6.406±0.850 x 10^6	4.726±0.673 x 10^6	<0.0001
	$N_{R\text{-}Spermatid}$	1.415±0.251 x 10^7	1.378±0.205 x 10^7	0.2597
	$N_{E\text{-}Spermatid}$	1.104±0.157 x 10^7	1.005±0.187 x 10^7	0.0004
	$N_{Sertoli}$	1.958±0.421 x 10^5	1.188±0.248 x 10^5	<0.0001
	$N_{Peritubular}$	2.490±0.484 x 10^6	2.373±0.610 x 10^6	0.2237
	N_{Leydig}	1.910±0.495 x 10^6	1.253±0.316 x 10^6	<0.0001

Semczuk-Sikora et al., 2010). Also, VPA levels in umbilical cord serum are up to 5 times higher than maternal levels (Albani et al., 1984). Therefore, when exposed to doses that can reach higher concentrations, this would generate a greater degree of alteration in testicular development. Our study group had previously demonstrated in the same model that VPA causes morphological alterations in the development of the spinal cord of embryos and fetuses of the same ages, where both neuroblasts and motor neurons were smaller and less defined nucleolus (Conei et al., 2016).

Meanwhile, in humans it has been documented that chronic consumption generates endocrine disruption, greater quantity of morphologically abnormal sperm, lower motility, smaller testicular volume and reduced fertility (Isojärvi et al., 2004; Hamed et al., 2015). Although a hormonal analysis was not performed in the present study, the morphological alterations present in the litters of mice are similar to those presented by patients treated with VPA.

A possible explanation for the reproductive alterations caused by VPA is due to the apoptotic effects it has on testicular tissue. In this regard, it is known that apoptosis occurs normally in the first pubertal spermatogenetic wave to maintain a physiological number of germ cells (Ohta et al., 2004), as it has also been shown that VPA has pro-apoptotic effects in testicular cells, where it has been seen to increase apoptotic cell counts and p53 immunoreaction (Cansu et al., 2011). This cell death may be due to the imbalance between endogenous antioxidants at the testicular level such as glutathione peroxidase, glutathione reductase and the generation of free radicals, explaining, in turn, a hormonal imbalance that eventually affects gonadal development and spermatogenesis (Aitken and De Iuliis, 2010). In mice, it has been seen that germ cells exposed to VPA generate an increase in lipid peroxidation and a decrease in antioxidant enzymes (Khan et al., 2011).

Furthermore, it is also known that one of the pharmacological actions of VPA is the inhibition of HDACs (Ghodke-Puranik et al., 2013), increasing the expression of genes involved in apoptosis (Phiel et al., 2001) and it can even act as an antitumor agent, increasing the efficacy of antineoplastic therapies by promoting the selective destruction of tumor cells (Bradbury et al., 2005; Zhu et al., 2017). This is of important consideration since HDAC6-specific deacetylase activity is observed in spermatogonia from 6-day-old mice, in pachytene spermatids, round and elongated spermatids from adult mice (Hazzouri et al., 2000) and is essential for sperm motility and functionality from birth to adulthood (Zhang et al., 2008; Verma et al., 2017). Also, the inhibitory activity of HDACs induced by VPA causes the acetylation of histones H3 (Detich et al., 2003), and histopathological alterations with morphologically abnormal round spermatids shed as well as marked apoptosis in spermatocytes, determining that adequate regulation of acetylation levels of histone H3 is important for the differentiation of spermatids (Fenic et al., 2008; Dai et al., 2015). Therefore, we hypothesize that the changes caused by VPA throughout testicular development are due to its action on DNA methylation. In the present study, the hormonal effects of VPA were not investigated, so the alterations produced in testicular development cannot be directly related to hormonal changes associated with VPA.

Conclusions

Prenatal exposure to VPA, a drug for the treatment of epilepsy, causes long term effects in the offspring of mice throughout testicular development, decreasing volumes of testicular tissue and histopathological alterations, with a lower number of spermatogenic, sustentacular Sertoli, peritubular and interstitial Leydig cells.

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