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BIOMEDICAL SCIENCES

Olanzapine during lactation: impact on testicular morphometry and endocrine parameters in adult wistar rats

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Abstract: Olanzapine (OLZ) is an antipsychotic medication used to treat postpartum psychiatric symptoms. It aimed to evaluate the effects of administering OLZ to lactating rats on testicular parameters of adult Wistar rats. Mothers received 2.5, 5 or 10 mg/kg until weaning. Adult male rats showed decrease in body weight, weight of testes, epididymis, prostate, seminal gland and gonadosomatic index when higher doses of OLZ were administered. Testicular volumetric parameters, as well as the length of seminiferous tubules, were also reduced in animals treated with the highest doses of OLZ. The diameter of the seminiferous tubules and the height of the seminiferous epithelium were reduced. There was also a relevant decrease in the population of Sertoli cells and a relevant reduction in the volume of individual Leydig cells. Histopathological analysis of the testes showed lesions compatible with testicular degeneration in rats treated with the highest dose of OLZ. There was a significant reduction in plasma testosterone levels in all treatments. It is noted, therefore, that the adverse impact on the testes of the highest doses of the drug during the neonatal period persisted into adulthood, with the dose of 2.5 mg/kg of OLZ proving to be safer than the others.

Key words: Mental disorders, breast-fed infant, reproduction, spermatogenesis, testosterone, prolactin.

INTRODUCTION

Infertility and sexual dysfunction in patients suffering from mental disorders, such as schizophrenia and bipolar disorder are clinical manifestations frequently reported in both sexes (Bobes et al. 2003, Montejo et al. 2021). However, the relationship between reproduction, mental illness and antipsychotics needs more attention or recognition regarding medical relevance. Paradoxically, mental illnesses have been growing in recent years (Steel et al. 2014, Auerbach et al. 2018, Szőllősi et al. 2022). The incidence of psychiatric disorders is more frequent during the postpartum period, with an incidence of 10% to 20% in new mothers (Manouilenko et al. 2018, Chithiramohan & Eslick 2023), making of great relevance to look for the possible effects of the maternal treatment to the descendent.

Antipsychotic drugs prescribed to treat psychiatric symptoms during the postpartum period are secreted into breast milk (Gardiner et al. 2003, Schoretsanitis et al. 2020). Because breast-feeding is crucial to infant development, it is important to select a medication that poses the fewest adverse consequences. In this regard, Olanzapine, Quetiapine, and Risperidone are cited as safe, although

monitoring is recommended (Klinger et al. 2013). It has been demonstrated that despite Olanzapine (OLZ) is excreted in the breast milk, the breast-fed infant had very low OLZ concentrations, not resulting in noticeable adverse effects (Gardiner et al. 2003, Manouilenko et al. 2018, Schoretsanitis et al. 2020).

Olanzapine is an atypical second-generation antipsychotic, who bind to serotonin (5-HT) and dopamine D(2) receptors, with a lower incidence of extrapyramidal side effects compared to first-generation antipsychotic drugs (Kuroki et al. 2008). Hyperprolactinemia detaches as a common adverse effect of antipsychotic medication, and it is known that elevation of prolactin levels caused by antipsychotics may contribute to sexual dysfunction (Montejo et al. 2015, Drobnis & Nangia 2017). Evidence seems to indicate that OLZ can induce a lower prolactin elevation, compared to other second and first-generation antipsychotic drugs (Peuskens et al. 2014).

Dopaminergic receptors were detected in rat testis, suggesting a probable interaction between the nervous and reproductive systems (Hyun et al. 2002, Otth et al. 2007). In fact, it was demonstrated in adult rats that OLZ caused disturbances in spermatogenesis, by disrupting the organization of the seminiferous epithelium, alterations in androgen-dependent organs and in serum testosterone, LH, FSH and in antioxidant enzymes of the testes; as well a decrease in normal sperm morphology; which may lead to male subfertility (Bringel et al. 2013, Ardıç et al. 2021). Toxic effects were also observed in testes of prepuberal animals whose mothers had been treated with OLZ during lactation period, with a reduction on parameters indicatives of sperm production, as well in serum testosterone, and an increase in prolactin levels (Lima et al. 2023). However, OLZ effects in newborns during the postnatal development period, and its repercussion in adult life, are poorly studied (Mishra & Mohanty 2010, Viswanathan et al. 2021).

Considering that antipsychotic drugs can cause changes in the function of the pituitary-gonadal axis, including disturbances in the gonadal development and hormones (Smith et al. 2002, Mishra & Mohanty 2010); and the lack of information regarding the impact to spermatogenesis of the drug administration in breastfeeding period; the present study aimed to evaluate the effects of OLZ administrated during the lactation period and its repercussions on testicular and endocrine morphometric parameters in adult Wistar rats.

MATERIALS AND METHODS

Experimental design

Twelve pregnant Wistar rats (230-280g) (*Rattus norvegicus*, var. Albinus) from the vivarium of the Departamento de Morfologia e Fisiologia Animal da Universidade Federal Rural de Pernambuco (UFRPE), Recife-PE, Brazil, were kept in a controlled environment at 22 °C and 50% humidity, with a 12 h light–dark cycle. Standard pellet food (Labina Purina) and water were available *ad libitum*. The dams received a daily dose of Olanzapine (OLZ) diluted in saline solution by gavage, during 21 days until weaning, and the females of control group received just vehicle for the same period. The following experimental groups were established: control (NaCl 0.9%) (n = 3), OLZ 2.5 mg/kg (n =3), OLZ 5 mg/kg (n =3) or OLZ 10 mg/kg (n =3).

To compose experimental groups, the neonatal rats were sexed after delivery. Twenty-four male rats from the offspring were randomly chosen to compose the same experimental groups related to

the treatments received for the dams: control (NaCl 0.9%) (n = 6), OLZ 2.5 mg/kg (n =6), OLZ 5 mg/kg (n =6) and OLZ 10 mg/kg. The puppies were maintained with the dams until the weaning, and they received Olanzapine via breastfeeding from the 1st day until the 21st postpartum day. At 90 days, all animals were submitted to euthanasia protocols to collect blood and tissue samples.

The experimental protocol was approved by the Ethics Committee of the Animal Wealfare of the Universidade Federal Rural de Pernambuco (CEUA/UFRPE- Protocol nº 23082008993/2013; Registration nº. 073/2013-CEUA/UFRPE).

Tissue perfusion and weight of androgen-dependent organs

At 90 days, all male rats were heparinized (125 IU/100g of body weight) and after 15 minutes, anesthetized intramuscularly with a combination of ketamine (50 mg/kg) and xylazine (5 mg/kg). Subsequently, a blood sample was taken from vena cava sinus and after clotting and centrifugation; the recovered serum was stored at -20 °C until further analysis for testosterone and prolactin measurement. An intracardiac perfusion was performed using a 0.9% NaCl solution plus heparin (500 IU l–1) and sodium nitroprusside (100 mg l–1; Sigma) for 5–10 min. After that, all rats were perfused using 4% glutaraldehyde (Vetec) in sodium phosphate buffer (0.01 M, pH 7.2) for 15 min. After fixation, the testes, epididymis, seminal gland and prostate were removed and weighed. The weighing of these androgen-dependent organs was done using a BEL Engineering scale (Mark 500/BRA) with 0.001 g precision.

Testicular histomorphometry and histopathology

Testicular samples were cut to a 2 mm-thickness. These sections remained immersed for 2 h in phosphate buffer and then dehydrated in an alcohol series for embedding in plastic resin composed of glycol methacrylate (Leica). Histologic sections of 4-µm thickness were stained with 1% toluidine blue–sodium borate and analyzed morphologically and morphometrically. Morphometric and quantitative analyses of testicular components were performed as described by Bringel et al. (2013), using Leica DM500E microscope (Germany).

The gonadosomatic index (GSI) was calculated according to the following formula: GSI (%) = [testicular weight / body weight] x 100 (Bringel et al. 2013, Lima et al. 2023).

Volume of testicular tissue components

The volume density of testicular components was obtained by evaluation of 15 photomicrographs randomly selected from the testicular parenchyma with 400X magnification. All photomicrographs were evaluated by ImageJ software (Version 1.52v) using point counting by systematic allocation through a micrometer reticule with 441 intersection points, totaling 6615 points counted for each animal. The volume of each component of the testis, expressed in microliters (µL), was established from the product of the volume density of testicular components (%) and testicular liquid weight, calculated in milligrams (mL). The value of testicular liquid weight was obtained by subtracting 6.5% (relative to the albuginea) of the testicular gross weight (Bringel et al. 2013). As the density of testis is approximately 1.03–1.04 (Johnson et al. 1981), the testis weight was considered equal to its volume (Silva et al. 2019, Bringel et al. 2013).

Tubular diameter, seminiferous epithelium height and seminiferous tubule total length

The mean diameter of 30 round seminiferous tubules in cross section, randomly selected from the testicular parenchyma per animal, were measured on the ImageJ software, using a linear reticle micrometer at 100× magnification. The same seminiferous tubules were used to measure the height of the seminiferous epithelium, measuring from the basal membrane to the tubular lumen. The tubular diameter and epithelium height in each tubule were performed by the average of two diametrically opposite measurements. The total length of the seminiferous tubules (TLST) per testis, expressed in meters (m), was obtained by dividing the seminiferous tubule absolute volume (STAV) by the squared radius of the tubule (r^2) and the π-value, according to the formula: TLST (m) = [STAV / πR^2] (Bringel et al. 2013, Dias et al. 2022, Lima et al. 2023).

Sertoli cells counting

The corrected number of Sertoli cells was estimated at stage VII of the seminiferous epithelium cycle, classified according to the acrosomal method (Russell et al. 1990). Counts of Sertoli cells nucleoli were performed, using 10 cross-sections of seminiferous tubules per animal. The Sertoli cells count (SCC) were corrected for nucleolar diameter and histological section-thickness. The crude counts (CC) were corrected for section thickness (S) and the mean nuclear or nucleolar diameter (ND), according to the equation of Abercrombie (1946) modified by Amann & Almquist (1962):

$$GSCC = CC \times \{S / [S + \sqrt{(\frac{ND^2}{2}) - (\frac{ND^2}{4})}]\}.$$

The mean nucleolar diameters were measured using a linear reticle micrometer at 1000× magnification (ImageJ Version 1.52v Software). The number of Sertoli cells per testis (NSCT), also called the Sertoli cell population, was determined from the corrected counts of Sertoli cells per tubule in cross-section (NSC), section thickness and the total length of seminiferous tubules (TLST), according to the formula: NSCT = [(TLST x NSC) / S].

Leydig cell morphometry

Initially, the average nuclear diameter of 50 Leydig cells was determined after capturing images at 1000x magnification (DM500E-Leica, Germany) and analyzed with the LAZ EZ 11.4 software. The volumetric density of Leydig cells (%) was determined by 1000 points counted over its nucleus and cytoplasm. Photomicrographs were evaluated by ImageJ software, using point counting by systematic allocation through a micrometer reticule with 441 intersection points. The individual volume of the Leydig cell was determined with mathematical models, using the values of nuclear diameter and the volume of the sphere: $4/3 \times \pi R^3$.

The cytoplasm (LcCV) and Leydig cell volumes (LcV) were obtained with the following formulas: LcCV = [(CtD (%) x NV) / ND (%)] and LcV = NV + LcCV; where LcCV = Leydig Cell Cytoplasm Volume; CtD = Cytoplasm Density (%); NV = Nuclear Volume of Leydig cell; ND = Nuclear Density (%); and LcV = Leydig cell volume (Dias et al. 2020, 2021).

The population of Leydig cells per testis was calculated from the individual volume of the Leydig cell (LcV) and the total volume of these cells in the testis (TVLc), according to the formula: [TVLc /

LcV]. This value was divided by the gonadal weight to estimate the number of Leydig cells per gram of testis (Bringel et al. 2013, Lima et al. 2023).

Testicular histopathologic evaluation

Testicular histological slides were examined qualitatively for histopathological alterations as a result of the treatments employed, and it were evaluated the presence of normal or altered morphology of tubular and intertubular compartments, presence of vacuolization on germ and somatic cells within the seminiferous tubules, germ cell loss, multinucleated cells, degeneration/ apoptosis of germ cells, germ cell exfoliation, necrosis and disorganization of tubular and intertubular contents (Lanning et al. 2002, Bringel et al. 2013).

Plasma testosterone and prolactin levels

The blood serum samples were kept in ependorffs at -20°C. At the moment of analysis, the samples were thawed at room temperature, homogenized and centrifuged at 3000 G for 10 minutes. Quantitative determination of these hormones was performed using the chemiluminescence technique (Beckman Coulter Access 2) with an absorbance reading at 405 nm.

Statistical analysis

A Shapiro–Wilks test was used to check the normality of the obtained data. Subsequently, depending on the normal trend of the results, parametric or nonparametric tests were used. For parametric data, it was performed analysis of variance (ANOVA) followed by a Tukey–Kramer post-hoc test. For nonparametric data, Kruskal–Wallis followed by Dunn's test was performed. Differences were considered statistically significant at a probability level of 5% ($p < 0.05$). GraphPad Prism software (Version 5.0) was used to elaborate graphics according to results obtained. The statistical treatment was designed with a significance level of $p < 0.05$.

RESULTS

Body weight, testicular weight and gonadosomatic index

The results of body weight, testes and androgen-dependent organs weights, and GSI of adult rats breastfeed by dams treated with different doses of OLZ can be observed in Table I. Body weight showed a statistical increasing in the groups treated with 5 mg/kg and 10 mg/kg of the drug, compared to the control and 2.5 mg/kg groups. Testis weight and testicular liquid weight of the 10 mg/kg groups showed a significant reduction in these parameters related to the other groups. Adult rats breastfeed by dams treated with the highest doses of OLZ, especially 10 mg/kg, had GSI reduced when compared to the control and 2.5 mg/Kg groups.

The epididymal and prostatic weights were reduced in animals treated with 10 mg/kg of OLZ compared with all other experimental groups. Adult rats breastfeed by dams treated with 5 mg/kg and 10 mg/kg OLZ had a reduction in the seminal gland weights compared to the others experimental groups.

Testicular histomorphometry

Table II shows the volume of testicular parenchyma components and histomorphometric parameters of adult rats breastfeed by dams treated with different doses of OLZ. Seminiferous tubules and seminiferous epithelium volume were significatively reduced in adult rats breastfeed by dams treated with 10 mg/Kg of OLZ compared to the control and 2.5 mg/Kg groups. Tubular lumen volume of adult rats was not difference in the experimental groups. However, tunica propria volume was higher in adult rat of group treated with 10 mg/Kg compared to the untreated animals ($p = 0.0001$).

Leydig cell volume was reduced in adult rats treated with 10 mg/Kg related to the others experimental groups. On the other hand, connective tissue cell volume ($p = 0.010$) and lymphatic space volume had significant increases in adult rats treated with the same dosage compared to other groups.

Adult rats breastfeed by dams treated with all dosages of OLZ had a reduction ($p = 0.004$) in the seminiferous tubule's diameter compared to the control group. Likewise, seminiferous epithelium height showed a progressive reduction related with this antisquizophrenic drug dose ($p<0.0001$). Seminiferous tubules total length was reduced in 10 mg/kg-treated animals compared to all the other groups. The breastfeed exposition to OLZ also impacted on Sertoli cells population, where the higher doses of the antipsychotic drug showed Sertoli cell population reduction compared with unexposed animals (Table II).

The Leydig cell nuclear diameter (μm) and individual volume (μm^3) were reduced in adult rats breastfeed by dams treated with all dosages of OLZ in relation to the unexposed animals ($p < 0.0001$), with a reduction dose dependent. On the other hand, no differences were observed in the Leydig cells population per gram of testis among the different experimental groups.

Testicular histopathologic evaluation

According to histopathologic evaluation of the testis, it was observed changes in seminiferous tubules compatible with testicular degeneration when the animals were exposed to OLZ through

Table I. Body weight, testes and androgen-dependent organs weight, and gonadosomatic index (GSI) data of adult Wistar rats of 90 days-old untreated (control) or treated for 21 days with different doses of Olanzapine through breast milk.

	Experimental groups			
Parameters	**Control**	**2.5 mg/kg**	**5 mg/kg**	**10 mg/kg**
Body weight (g)	260.17 ± 6.17[a]	273.20 ± 7.02[a]	288.00 ± 15.9[b]	302.83 ± 7.50[b]
Testes weight (g)	1.57 ± 0.13[a]	1.53 ± 0.10[a]	1.41 ± 0.05[a]	1.21 ± 0.22[b]
Testicular liquid weight (g)	1.46 ± 0.12[a]	1.43 ± 0.09[a]	1.34 ± 0.05[a]	1.13 ± 0.21[b]
Epididymis (g)	0.61 ± 0.13[a]	0.60 ± 0.14[a]	0.62 ± 0.05[a]	0.41 ± 0.04[b]
Prostate (g)	0.68 ± 0.17[a]	0.50 ± 0.16[a]	0.56 ± 0.12[a]	0.44 ± 0.08[b]
Seminal gland (g)	1.98 ± 0.43[a]	1.45 ± 0.18[a]	1.13 ± 0.13[b]	1.14 ± 0.22[b]
GSI (%)	0.58 ± 0.05[a]	0.56 ± 0.04[ab]	0.49 ± 0.04[b]	0.40 ± 0.02[c]

Values are expressed as mean standard error of the mean. Different letters in the same line indicate statistically significant difference among groups, at $p<0.05$.

breast milk (Figure 1). Testicular parenchyma in animals treated with all dosages of the drug showed seminiferous epithelium with germ cell desquamation, multinucleated giant cells, intracytoplasmic vacuolization on Sertoli cells, necrotic and apoptotic germ cells in tubular lumen. The testicular damage was pronounced in breast-feed rats by dams who received the highest dose of OLZ during the 21 postnatal days.

Plasma testosterone and prolactin levels

The testosterone and prolactin levels are shown in Figure 2. It was observed a reduction in testosterone level in rats treated with all dosages of OLZ, in a dose-dependent way ($p<0.0001$). On the other hand, the prolactin plasma level was higher in breastfeed rats by dams that received 5mg/Kg and 10mg/Kg of Olanzapine during lactation period ($p = 0.00031$).

DISCUSSION

In the present study, the effects of different doses of OLZ via lactation on testicular parameters of adult Wistar rats were evaluated, and important repercussions on testicular and endocrine parameters were evidenced in adult animals; especially with investigation of body weight, weight of testes and

Table II. Histomorphometric parameters of adult Wistar rats of 90 days-old untreated (control) or treated for 21 days with different doses of Olanzapine through breast milk.

	Experimental groups			
Parameters	**Control**	**2.5 mg/kg**	**5 mg/kg**	**10 mg/kg**
Seminiferous tubule (µL)	1.31 ± 0.13^{a}	1.25 ± 0.08^{a}	1.20 ± 0.05^{ab}	1.00 ± 0.19^{b}
Seminiferous epithelium (µL)	1.10 ± 0.09^{a}	1.04 ± 0.08^{a}	0.99 ± 0.06^{ab}	0.83 ± 0.17^{b}
Tubular lumen (µL)	0.16 ± 0.04	0.15 ± 0.02	0.16 ± 0.06	0.14 ± 0.03
Tunica propria (µL)	0.028 ± 0.003^{a}	0.034 ± 0.005^{ab}	0.035 ± 0.007^{ab}	0.042 ± 0.007^{b}
Leydig cells (µL)	0.031 ± 0.005^{a}	0.024 ± 0.003^{a}	0.021 ± 0.005^{a}	0.020 ± 0.001^{b}
Connective tissue cells (µL)	0.006 ± 0.002^{a}	0.006 ± 0.003^{a}	0.006 ± 0.002^{a}	0.010 ± 0.003^{b}
Blood vessel (µL)	0.055 ± 0.049	0.044 ± 0.048	0.030 ± 0.003	0.017 ± 0.013
Lymphatic space (µL)	0.08 ± 0.02^{a}	0.07 ± 0.03^{a}	0.10 ± 0.01^{a}	0.13 ± 0.01^{b}
Tubular diameter (µm)	330.20 ± 15.60^{a}	288.10 ± 27.84^{b}	289.30 ± 10.59^{b}	287.30 ± 24.69^{b}
Epithelium height (µm)	131.43 ± 11.87^{a}	94.83 ± 7.28^{b}	86.15 ± 7.89^{b}	80.42 ± 16.04^{b}
Seminiferous tubules total length (m)	19.66 ± 1.70^{a}	17.14 ± 1.67^{a}	17.99 ± 1.55^{a}	13.22 ± 1.50^{b}
Sertoli Cell Population (x10^{7})	5.46 ± 1.28^{a}	4.40 ± 1.87^{ab}	3.88 ± 1.08^{b}	3.09 ± 2.70^{b}
Leydig cell nuclear diameter (µm)	9.03 ± 0.49^{a}	7.13 ± 0.44^{b}	6.99 ± 0.66^{b}	6.85 ± 0.34^{b}
Leydig cell individual volume (µm^{3})	942.2 ± 120.1^{a}	514.0 ± 101.8^{b}	460.7 ± 131.1^{b}	452.7 ± 70.6^{b}
Leydig cell population/gram of testis (x10^{7})	3.35 ± 1.00	2.64 ± 1.67	3.73 ± 1.18	4.19 ± 1.40

Values are expressed as mean standard error of the mean. Different letters in the same line indicate statistically significant difference among groups, at $p<0.05$.

androgen-dependent organs, qualitative and quantitative evaluation of spermatogenesis, as well in plasma levels of testosterone and prolactin.

Body weight, testicular weight and gonadosomatic index

The body weight of the animals in this study was directly influenced by OLZ administered to the mothers during the lactational period. Weight gain over a short period of exposure to antipsychotic therapy with OLZ is frequently reported in the literature when it is administered orally (Shobo et al. 2011, Davey et al. 2012, Zhang et al. 2010). In our study, this effect was observed, even though the animals were exposed through breastfeeding, and not through direct oral administration; so that our

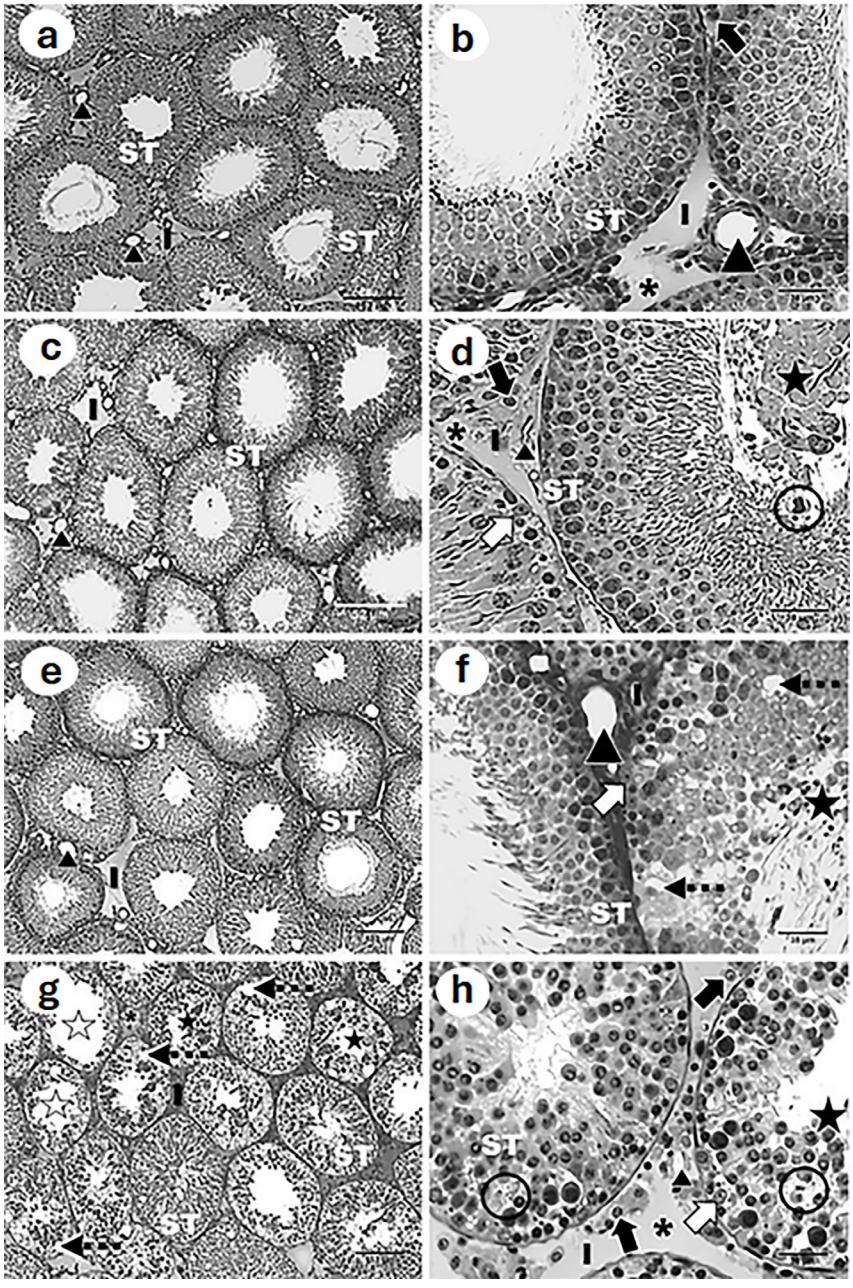

Figure 1. Testicular cross sections of adult Wistar rats treated during 21 days with different doses of Olanzapine through breast milk and evaluated at 90 days of age. a-b: Control (Untreated); c-d: 2.5 mg/kg of Olanzapine; e-f: 5 mg/kg of Olanzapine. g-h: 10 mg/kg of Olanzapine. ST = Seminiferous Tubules; I = Intertubule; Arrowhead = Blood vessel; * = Lymphatic space; Dark arrow = Leydig cell; White arrow = Sertoli cell. Details: In 2b it is shown seminiferous epithelium with preserved morphology. In 2d it is shown desquamated germ cells within the tubular lumen (black star), some of them in the death process (circle). In 2f it is shown seminiferous epithelium vacuolization (dashed arrow) and germ cells desquamation (black star). In 2g-h it is shown seminiferous epithelium vacuolization (dashed arrow), seminiferous tubules with germ cell reduction (white star), desquamated cell within the tubular lumen (black star), and germ cell loosening (circle). Bars = a, c, e, g: 53 µm. b, d, f, h = 38 µm.

results allow us to confirm the transfer of OLZ through breast milk, as reported in several studies (Gardiner et al. 2003, Manouilenko et al. 2018, Schoretsanitis et al. 2020).

All reproductive organs evaluated in this study showed a reduction in weight when the tested drug was administered, similarly to what was observed in adult animals when they were exposed to other psychotropic drugs during lactation (Vieira et al. 2013, Monteiro Filho et al. 2014). This finding deserves attention, since the evaluation of organ weights in toxicology studies is an integral component in the assessment of pharmaceuticals, chemicals, and medical devices (Sellers et al. 2007).

The testicular weight is a parameter of major relevance for evaluating the spermatogenesis, since it correlates directly with sperm production (França & Russell 1998, Condorelli et al. 2013). In our study, there was a significant reduction in gross and net testicular weights, and in the gonadosomatic index of animals treated via breast milk with OLZ, similar to that observed in other studies where rats were exposed to the same drug, both by oral administration to adult animals (Soliman et al. 2014) and through breastfeeding, with impacts on the weight of the developing gonad (Lima et al. 2023); indicating a possible adverse effect of this antipsychotic on male reproductive functions.

Epididymal, prostatic and seminal gland weights were reduced, especially at higher doses of the antipsychotic drug, as a direct effect of the reduction in plasma testosterone levels face to the treatments used here. It is reported that the reduction in epididymal weight is indicative of decreased spermatogenesis in the testis, reflected in the reduction in the number of spermatozoa in the epididymis (Creasy 2003, Mouro et al. 2018, Manoel et al. 2022, Dias et al. 2023). Reduction in plasma testosterone levels were also observed when the same antipsychotic was administered orally to adult animals (Bringel et al. 2013, Ardıç et al. 2021), as well as when animals were exposed to OLZ through breastfeeding, leading to a reduction in this hormone already in the period of gonadal development (Lima et al. 2023); which justifies the alterations found in the testicles, epididymis, and prostate weights.

Prostatic secretion, together with the secretion of the seminal or vesicular glands, are a fundamental part of semen composition, being of essential importance in the cascade of events related to ejaculation and acquisition of the fertilizing capacity by the sperm (Verze et al. 2016, Noda & Ikawa 2019). Thus, the impact of OLZ on the reduction in weight of these testosterone-dependent

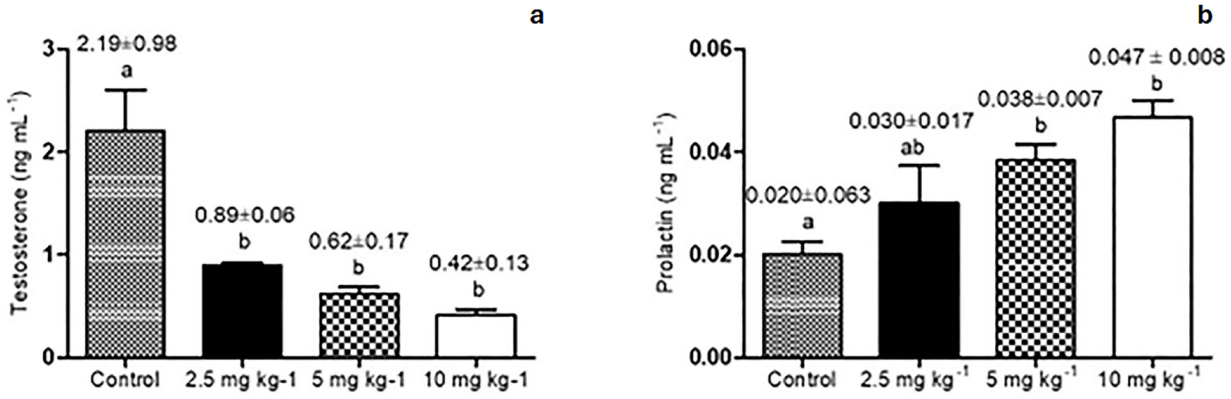

Figure 2. Testosterone (a) and prolactin (b) plasma level of adult Wistar rats control (untreated) or treated during 21 days with different doses of Olanzapine through breast milk. Values are expressed as mean standard error of the mean. Different letters in the columns indicate statistically significant difference from group at p<0.05.

organs reinforces a possible impairment on the reproductive capacity of the animals. This impact may even be related to testosterone bioavailability, since it is in the prostate that testosterone is converted to its most active metabolite, 5α-dihydrotestosterone, by the activity of the 5α-reductase enzyme (Lee & Janulis 1999, Swerdloff et al. 2017).

Testicular histomorphometry

The volume of the tunica propria increased considerably in the group of rats treated with the highest dose of OLZ, which characterizes impaired spermatogenesis. It is known that in testicular degenerative processes there is thickening of the tunica propria, characterized by stimulation of the deposition of extracellular matrix caused by the increase in the content of collagen fibers and glycosaminoglycans (Paniagua et al. 1991, Pinart et al. 2001). This may lead to a tubular sclerosis that hinders the action of testosterone produced by the Leydig cells on the cells of the seminiferous epithelium (Honoré 1978).

Administration of OLZ to neonates via breast milk also altered the liquid volume of Leydig cells in adult animals, as well as their cell volume and nuclear diameter, which had repercussions on the reduction of testosterone levels. These cells constitute the source of testicular androgens that are synthesized from a base molecule, the cholesterol, with the liquid volume of Leydig cells being a parameter related both to the cell population and to the steroidogenic activity of these cells. The relationship between the number and size of Leydig cells and the ability of these cells to produce testosterone is well known (França & Russell 1998, Bringel et al. 2013, Zirkin & Papadopoulos 2018).

The volume of cells and fibers of the connective tissue increased in the testis of adult rats that received the highest dose of OLZ via breast milk. Previous studies have associated this drug with macrophage infiltration and elevation of pro-inflammatory markers in rodents (Davey et al. 2012, Victoriano et al. 2010). It is also known that testicular degenerative processes can induce an increase in cells and fibers of connective tissue as a result of testicular elevation of plasma TNF-α, IL-1β, iNOS and IL-1β, which can be related to hormonal dysfunction and low intra-testicular testosterone level, processes relevant to male infertility (Yildirim et al. 2019).

As well for the increase in the volume of connective tissue, the increase in the volume of lymphatic space, also observed in the testicular intertubular tissue of animals that received the highest dose of OLZ via breast milk, may be related to the decrease in seminiferous tubules volume, observed in the animals of this group (França & Russell 1998). The total length of the seminiferous tubule, tubular diameter, epithelium height and Sertoli cell population were also influenced by the use of the antipsychotics during breastfeeding. These data corroborate those obtained by Mishra & Mohanty (2010), who observed a reduction of 16-17% in the diameter of the seminiferous tubules in neonates submitted to treatment with OLZ via breast milk. Likewise, Bringel et al. (2013) observed this effect on tubular diameter and height of seminiferous epithelium in adult rats exposed to OLZ.

The number of Sertoli cells in the testis established before puberty determines the magnitude of sperm production in sexually mature animal (Hess & França 2007). It has been considered that these key testis somatic cells stop dividing during early pre-pubertal phase, between around 10 to 20 days after birth respectively in mice and rats, being after that under physiological conditions a stable and terminally differentiated population. However, undifferentiated Sertoli cells were identified in rats with 36 days old capable of growing the seminiferous tubules length (Figueiredo et al. 2016). In the present study, OLZ via breast milk, during the main period of Sertoli cell proliferation,

produced permanent alterations in the testicular morphometric parameters directly related to this cell population, significantly decreasing its population, which is a finding of great relevance and great impact on testicular function, given the importance that Sertoli cells plays for spermatogenesis (Russell et al. 1990, Sharpe 1994).

Testicular histopathologic evaluation

In regulatory toxicology studies, histopathological examination is accepted as one of the most sensitive biomarkers to detect toxicants' adverse reproductive effects. The morphometric analysis of our study was supported by the histopathological findings, and the related pathologies observed are crucial indicators of OLZ-induced germ cell damage or reproductive toxicity (Creasy 2003, Ardiç et al. 2021). Most of the histopathological changes found in our study occurred with all dosages of OLZ, being higher at the highest dosages.

While cells in death process were observed at 2.5 mg/kg group, this wasn't observed at the higher dosages. Apoptotic cells were also observed by Lima et al. (2023) in pre-puberal rats that received OLZ on the same dosage, through breast milk; however, on that case the apoptotic cells were observed also when these animals were exposed to the highest dosages of the drug. Vacuolization in spermatogenic cells of adult animals treated with 5 mg/kg and 10 mg/kg of OLZ were also observed in adult and in pre-puberal animals that receive OLZ orally (Ardiç et al. 2021, Lima et al. 2023), and our results evidences the impact for testicular morphology of adult animals that received the drug by breast milk.

Necrosis and loss of germ cells also were observed at the highest dosages. According to Lanning et al. (2002), necrosis and disorganization of tubular contents, including Sertoli cells, may be observed when there is evidence of acute inflammatory infiltrate around affected tubules, possibly caused by disturbance in hemodynamics or damage to the vascular endothelium leading to ischemic necrosis. Also like our findings, it is reported that the most common indicator of Sertoli cell degeneration are vacuolization and presence of exfoliated germ cells in the lumen. We found desquamated germ cells in seminiferous tubules at all dosages of OLZ administrated, similar to the observed in pre-puberal rats (Lima et al. 2023); which is an indicative of disruption of Sertoli/germ cell junctions, leading to loss of adhesion (Lanning et al. 2002).

Generally, disorganization, exfoliation, or degeneration of germ cells accompany vacuolization and swelling. It is known that any functional deficit of Sertoli cells probably leads to germ cell degeneration by interacting with toxic substances (Creasy 2001, Vidal & Whitney 2014, Ardiç et al. 2021), like founded here for OLZ.

Plasma testosterone and prolactin levels

The exposure of the animals in this study to OLZ through breast milk had a considerable impact on serum levels of testosterone and prolactin in adulthood, where the higher the dosage of OLZ, the lower the levels of testosterone and the higher the levels of prolactin, which was related to an important negative impact on the sperm production of the animals evaluated. The findings regarding the testicular histopathological analysis and the morphometry of the Leydig cells and Sertoli cells of the animals in this study, as previously demonstrated, allow inferring a relationship with the changes found in testosterone and prolactin levels (Ardiç et al. 2021).

As previously shown, the lactational exposure to OLZ interfered with the pituitary–testicular axis by prominently influencing blood levels of pituitary hormones prolactin and luteinizing hormone (LH), and adversely affecting testosterone levels and testicular histopathology (Mishra & Mohanty 2010). Our results corroborate the findings of Bringel et al. (2013) and Ardıç et al. (2021), who obtained a reduction in testosterone levels in adult rats treated orally with different doses of OLZ; as well as with the findings by Rosa et al. (2003), who showed the relationship between hyperprolactinemia and the reduction of testosterone in men.

The levels of follicularstimulating hormone (FSH), the main mitogenic factor of Sertoli cells (Singh & Handelsman 1996), may have been negatively influenced by the persistent increase in prolactin levels in adult rats that received OLNZ via breast milk, justifying the reduction in the population of these cells in the group that received the highest doses of the drug. It is known that prolactin increase can affect the hypothalamic-pituitary-gonadal axis (Smith et al. 2002) and induce hypogonadism because of inhibition of gonadotropinreleasing hormone (GnRH), FSH, LH, testosterone, and androgen binding protein (ABP); which results in morphological changes in testis as well as a delay in spermatogenesis (Katovich et al. 1985, Rosa et al. 2003), similar to our findings. Thus, it can be inferred that pharmacological manipulation by OLZ on the hypothalamic-pituitary-gonadal axis during the neonatal period resulted in permanent testicular and hormonal alterations in adult rats.

The main testicular alterations related with hyperprolactinemia are seminiferous epithelial disorganization, germ cell exfoliation, increased tubule wall thickness, and abnormal Leydig cell lipid content (Aleem et al. 2005). Most of them were also observed in our study, where testicular lesions were compatible with the decrease in testosterone and increase in prolactin levels, resulting in important changes on spermatogenesis. It is also important to know that the prolactin receptors are localized exclusively in the interstitial cell tissue (Charreau et al. 1977). Hyperprolactinemia experimentally induced produced structural changes in cells expressing the androgen receptor in the testis, epididymis and prostate (Słuczanowska-Głabowska et al. 2004), which also reinforces the possible reduction on the weight of these organs in the present study, as previously shown.

CONCLUSIONS

The adverse effects of OLZ in the hypothalamic-pituitary-gonadal axis during the neonatal period had repercussions on testicular hormonal function in adulthood, as well as on testicular morphometric parameters. These alterations are mainly related to the maintenance of elevated prolactin levels, even in the absence of OLZ administration in adult animals. Our results also allow us to conclude that the dose of 2.5 mg/kg of OLZ proved to be safer to be administered to lactating females, when considering the impacts on the spermatogenesis of the adult offspring.

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