Acute effects of \textit{para}-chloroamphetamine on testosterone and markers of apoptosis in seminiferous epithelium of prepubertal male rats

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\textbf{ABSTRACT}

Serotonin is a neurotransmitter that affects the secretion of gonadotropins and testosterone. In prepubertal male rats, serotonin has a stimulating role in testosterone secretion. Here, we used prepubertal male rats to study the effects of \textit{para}-chloroamphetamine (pCA) on circulating testosterone and gonadotropins and markers of apoptosis in germ cells from day 1 to day 5 post-treatment. The intraperitoneal administration of pCA induced a significant reduction in concentrations of hypothalamic serotonin and circulating testosterone, but gonadotropins were not affected. In the seminiferous epithelium of pCA-treated rats, increased number of germ cells positive to markers of apoptosis, concomitantly with alterations in morphology and the presence of multinucleated germ cells. Levels of testosterone were reduced starting from 1 day after pCA was administered. The time window between the administration of the pCA and collection of samples was sufficient to detect changes in testosterone levels, in contrast with a previous work where no changes were found. There was a possible relationship between the reduction of testosterone and an increase in the number of germ cells positive to apoptosis markers. However, the mechanism that links pCA-testosterone–germ cell positive to markers of apoptosis is unknown. Our outcomes support the view that pCA exposure during the prepubertal stage has an acute impact on testosterone levels and affects the structure and physiology of seminiferous epithelium.

\textbf{Abbreviations:} pCPA: \textit{p}-chlorophenylalanine; 5-HIAA: 5-hydroxyindoleacetic acid; 5-HT: 5-hydroxytryptamine; 5-MeO-DMT: 5-methoxy-N,N-dimethyltryptamine; AC: absolute control; HHTA: hypothalamus–hypophysis–testicle axis; MANOVA: multivariate analysis of variance; RIA: radioimmunoassay; TdT: terminal deoxynucleotidyl transferase; VH: vehicle group

\textbf{KEYWORDS}

\textit{p}-Chloroamphetamine; serotonin; male germ cells; testosterone; apoptosis

\textbf{Introduction}

The neurotransmitter serotonin (5-HT) regulates the physiology of the hypothalamus–hypophysis–testicle axis (HHTA) (Aragón et al. 2005; Ayala et al. 2015). The disruption of the serotonergic system leads to alterations at distinct levels of the HHTA, such as spermatogenesis or sex steroid production. Amphetamine derivatives are among substances that inhibit the serotonergic system at the central nervous system level (Booij et al. 2014; Ma et al. 2016); however, their effects on testicular physiology have not been well studied.

Serotonin affects the secretion of hormones such as gonadotropin-releasing hormone (Bhattarai et al. 2014; McArdle and Roberson 2015) and gonadotropins (LH and FSH) (Ayala 2009). It has been related to the \textit{in vitro} secretion of testosterone by Leydig cells (Tinajero et al. 1993; Frungieri et al. 1999). The effect of serotonin on testosterone secretion in rats appears to depend on the age of the animal, i.e., with stimulatory activity in prepubertal rats and inhibitory function in adults (Justo et al. 1989; Collin et al. 1996; Csaba et al. 1998; Piner et al. 2002; Ayala et al. 2015). For instance, (1) in prepubertal male rats (30 days old), with the elimination of innervation from the
dorsal nucleus of raphe toward the hypothalamus, decreased FSH levels and alteration of spermatogenesis were seen (Ayala et al. 2015); (2) in adult male rats, serotonin and FSH increased after administration of the serotonin precursor 5-hydroxytryptophan (Justo et al. 1989); and the intratesticular administration of serotonin inhibited the synthesis of testosterone (Csaba et al. 1998). In Leydig cells from adult rats cultured in vitro, serotonin binds to the 5-HT2 receptor, stimulating the release of the corticotropin factor inhibiting testosterone synthesis (Tinajero et al. 1993).

There is evidence that amphetamine derivatives affect the activity of the serotonergic system and impair the HHTA, notably the testicular function (Shishkina and Dygalo 2000; Aragón et al. 2005; Ayala 2009); for example, p-chlorophenylalanine (pCPA), administered intraperitoneally to male rats from 40 to 44 days-of-age, diminished the circulating concentrations of testosterone (Shishkina and Dygalo 2000). Previously, we reported that intraperitoneal administration of pCA to male rats of 30 days-of-age, and euthanized at 45- or 65-days-of-age, decreased the serotonin levels in the hypothalamus and greatly increased the presence of TUNEL-positive germ cells (Aragón et al. 2005). The pCA is an amphetamine derivative that binds to the serotonin transporter and inhibits serotonin uptake (García-García et al. 2015), decreases the concentration of serotonin and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) (Fuller 1992), and induces degeneration of serotonergic axon terminals in the forebrain (Jin et al. 2016).

Apoptosis of male germ cells is a natural phenomenon (Shaha et al. 2010; Xu et al. 2016; Bejarano et al. 2018) that can be induced by depriving the cells of gonadotropins or testosterone (Yao et al. 2012; Dimitriadiis et al. 2015). Recovery from germ cell loss induced by the suppression of gonadotropins or testosterone is achieved by administering the relevant hormone (Elkis et al. 2013; Dutta et al. 2017). Thus, gonadotropins and testosterone act as survival factors (Tapanainen et al. 1993; Aragón et al. 2007; Shaha et al. 2010). Intriguingly, in our previous work, neither testosterone nor FSH levels were modified (Aragón et al. 2005). In these reports, hormone levels were not assessed immediately (i.e., just after inhibition of the serotonergic system); thus, it is possible that subsequent compensatory mechanisms participated in elevating gonadotropin and testosterone levels.

The communication established by testosterone and receptors in the Sertoli cells is essential to maintain the number of spermatogonium, the tight junctions among Sertoli cells, meiotic cell division, spermatid differentiation, and adhesion of germ cells to Sertoli cells (Smith and Walker 2014; O’Hara and Smith 2015). Thus, dysregulation of the chemical communication in the seminiferous epithelium, induced by alterations in the serotonergic system, could lead to germ cell loss.

For rats, the window from 31 to 35 days-of-age is between the final juvenile and peripubertal stages (Ojeda et al. 1980). In this developmental window, the diameter of seminiferous tubules increases and spermatocytes progress toward the spermatid stage in response to the growing concentrations of testosterone (Picut et al. 2015) and testosterone receptors (Abd El-Meseeh et al. 2016). In this work, we hypothesized that dysregulation of the serotonergic system induced by pCA, at 30 days of age, reduced gonadotropins and testosterone, thus affecting germ cell development in the 1–5 days interval after administration of pCA. Our goal was to quantify circulating concentrations of gonadotropins and testosterone in prepubertal male rats on days 1–5 after treatment with pCA and to evaluate the presence of germ cells with apoptosis markers (caspase-7 and TUNEL) in the same time period.

**Results and discussion**

Previously, it was reported that dysregulation of the serotonergic system by pCA does not change the testosterone levels of male rats treated from 30 until 60 days-of-age (Aragón et al. 2005). In this study, we demonstrated that the concentrations of hypothalamic serotonin and circulating testosterone diminished significantly from pCA post-treatment days 1 to day 5. Reduced levels of testosterone were accompanied by changes in morphometry of seminiferous epithelium and an increased presence of germ cells positive to markers of apoptosis.

**Effect of the pCA on body weight and sexual organ**

For body and sex organ weight, a 3 treatment × 5 day MANOVA showed a highly significant multivariate main effect for treatment (Wilks’s λ = 0.687, p < 0.0001, η = 0.313) and day (Wilks’s λ = 0.462, p < 0.001, η = 0.538) (S1 Table, weight of organs). The testis weight in pCA group was significantly lower than controls on days 3, 4, and 5 (p < 0.0001).

**Concentration of serotonin and 5-HIAA in the hypothalamus and hypophysis**

For serotonin and 5-HIAA in hypothalamus, hypophysis, and gonadotrophins and sex steroids in serum, a 3
treatment × 5 day MANOVA showed a highly significant multivariate main effect for treatment (Wilks’s λ = 0.1100, p < 0.0001, η = 0.89) and day (Wilks’s λ = 0.0230, p < 0.0001, η = 0.977).

In the anterior and median hypothalamus, concentrations of serotonin and 5-HIAA of the pCA group were significantly lower than AC and VH groups on all days analyzed (Figure 1). Treatment with pCA reduced the concentration of serotonin in the hypophysis, on all days of analysis except day 3 (Figure 2).

The reduced concentration of serotonin, in the anterior and medium hypothalamus, from day 1 to day 5 after pCA treatment, was possibly the result of inhibition of tryptophan hydroxylase, as previously demonstrated (Sanders-Bush et al. 1972a, 1972b).

Concentrations of gonadotrophins and testosterone

The administration of pCA did not induce changes in concentrations of gonadotropin or progesterone, but testosterone concentrations were significantly lower than controls from days 1 to 5 after treatment (Figure 2).

In rats treated with pCA, the ‘gonadotropins’ levels were not modified significantly, as previously demonstrated (Aragón et al. 2005). In this work, the serotonin concentrations were not eliminated by pCA; thus, the remaining levels could be sufficient to activate ‘gonadotropins’ production. This idea is supported by the fact that other amphetamine, the 3,4-methylenedioxymethamphetamine (MDMA, ecstasy), could strongly affect serotonin levels (Stone et al. 1987). However, MDMA decreased the GnRH mRNA level without affecting the LH concentrations (Dickerson et al. 2008).

In this study, the prepubertal rats treated with pCA showed reduced levels of testosterone from day 1 post-treatment. Thus, the synthesis pathway of testosterone could be impaired by serotonergic communication, as previously reported: (1) in immature male rats, serotonin binding to the 5-HT2 receptor stimulates testosterone synthesis (Csaba et al. 1998); and (2) in Leydig cells from adult rats, exposure in vitro to amphetamine diminished the levels of testosterone and the activity of the steroidogenic enzymes (3β-hydroxysteroid dehydrogenase, 17z-hydroxylase/C17–C20 lyase, and 17-ketosteroid reductase) (Tsai et al. 1997). Unexpectedly, the progesterone concentration in the pCA group were lower than controls only on day 2. Currently, we do not know how to interpret this reduction of progesterone.

The low levels of serotonin in the anterior and medial hypothalamus, of rats treated with pCA, could help to explain the decreased levels of testosterone observed in these rats. The fact that the gonadotropin secretion was not modified suggests that some kind of neural information, originating from the hypothalamus, plays a stimulatory role in testosterone concentrations of 5-HT and 5-HIAA in anterior and medium hypothalamus. Boxplots of concentrations of 5-HT and 5-HIAA in anterior and medium hypothalamus from day 1 until 5. Values are medians (black circle in boxes) with 25–75% interquartile ranges (boxes); solid vertical lines indicate minimum and maximum values; hollow circles indicate outliers. *p < 0.05 versus VH and AC (orthogonal contrasts). AC: absolute control; VH: vehicle group; pCA: para-chloroamphetamine group; 5-HT: 5-hydroxytryptamine; 5- HIAA: 5-hydroxyindoleacetic acid. N = 8–10 rats per treatment/day.

![Figure 1](image-url)
Figure 2. The administration of pCA reduced the levels of testosterone but gonadotropins were unchanged. Boxplots of concentrations of 5-HT and 5-HIAA in hypophysis, and circulating concentrations of testosterone, progesterone, and gonadotropins from day 1 until 5. Values are means (red dot in boxes), medians (black line in boxes) with 25–75% interquartile ranges (boxes); solid vertical lines indicate minimum and maximum values; black dots indicate outliers. *p < 0.05 versus VH and AC (orthogonal contrasts). AC: absolute control; VH: vehicle group; pCA: para-chloroamphetamine group; 5-HT: 5-hydroxytryptamine; 5-HIAA: 5-hydroxyindoleacetic acid. N = 8–10 rats per treatment/day.
synthesis. There is communication between the hypothalamus and the testis (Lee et al. 2002; Selvage and Rivier 2003; Selvage et al. 2006; Lee and Lee 2014); thus, an effect of the systemic administration of pCA on neural communication, between the hypothalamus and testicle, which leads to decreased concentrations of testosterone, is possible. In the neural pathway, pituitary independence arises from a hypothalamic nucleus, such as the paraventricular, directly regulates Leydig cells’ function (Lee et al. 2002; Selvage and Rivier 2003; Selvage et al. 2006; Lee and Lee 2014). The testis receives its serotoninergic innervation via the spermatic nerve (Campos et al. 1993), and sectioning this nerve in prepubertal male rats decreased testosterone secretion, reducing mRNAs’ levels of 3β-hydroxysteroid dehydrogenase, and the LH receptor in Leydig cells (Huo et al. 2010). Neural communication arises from a hypothalamic nucleus, such as the paraventricular, and directly regulates the function of Leydig cells via the spinal nerve (Lee et al. 2002; Selvage and Rivier 2003; Selvage et al. 2006; Lee and Lee 2014). However, how the pCA directly affects the neural communication between the hypothalamus and testicle in prepubertal rats is unknown.

**Effects of pCA on the seminiferous tubules**

Early, we described germ cell loss and the presence of TUNEL positive germ cells, after the subchronic treatment of juvenile rats with pCA (Aragón et al. 2005). In this work, we observed the typical structure of seminiferous epithelium and complete cellularity in control groups (Figure 3(A,B)), but seminiferous epithelium of rats treated with pCA was disrupted. In animals treated with pCA, the area of seminiferous tubules and the area of the seminiferous epithelium was significantly lower than in controls ($p < 0.0001$), although the lumen of the same tubules was greater ($p < 0.0001$) (Table 1 and inset at 400X of Figure 3(A)). The germ cell loss changed the form of the seminiferous tubules making them less round ($p < 0.0001$) (Table 1 and Figure 3(A,B)). Multinucleated giant cells were observed in the lumen.

![Figure 3. The administration of pCA induced an increase in the presence of TUNEL-positive and caspase-7-positive germ cells, and multinucleated giant cell cells. Representative micrographs of seminiferous epithelium from control or treated rats are presented. In each case, the same region was captured at several magnifications. In each row, the left column shows composite micrographs at 200X (lower) and 400X (upper) (see the letter size for clarity); in the right column shows a micrograph taken at 1000X. A corresponds to the labelling of TUNEL; in the case of pCA there is a clear increase of TUNEL-positive germ cells (brown cells). Note the relative size and form of seminiferous tubules in controls and pCA. B corresponds to the immunohistochemistry of caspase-7 active; in the case of pCA there is a clear increase of active caspase-7-positive germ cells (brown cells). Severe degeneration of seminiferous tubules (arrowheads) is observed in A and B. In C, there are representative micrograph of seminiferous tubules of the group treated with pCA, processed by TUNEL and counterstained by hematoxylin. Multinucleated giant cells are pointed up by arrows; note the numerous nuclei into each giant cell. For A and B, the asterisks in each row of micrographs indicate the seminiferous tubules which were magnified. Scale bar = 20 μm. Spc: spermatocyte; AC: absolute control; VH: vehicle group; pCA: para-chloroamphetamine group. The magnification in C was 400X.](image-url)
of seminiferous tubules of rats treated with pCA (Figure 3(C)).

Markers of apoptosis increased in the seminiferous tubules of rats treated with pCA. The percentage of seminiferous tubules with one or more TUNEL-positive germ cells was greater in the pCA group than in controls ($p < 0.0001$) (Table 2). TUNEL-positive germ cells were observed in controls and pCA-treated groups; there were no significant differences between controls ($p < 0.0001$). However, the number of TUNEL-positive germ cells per seminiferous tubule was significantly greater in animals treated with pCA ($p < 0.0001$) (Table 2). The TUNEL-positive germ cells were commonly located near spermatocytes. Seminiferous tubules expressing active caspase-7 were significantly more common in rats treated with pCA ($p < 0.0001$) (Figure 3(B)), and the number of germ cells expressing active caspase-7 per seminiferous tubule was greater ($p < 0.0001$) (Table 2). We did not observe a conspicuous difference in the location of germ cells positives to TUNEL or caspase 7 in the seminiferous epithelium.

LH (Marathe et al. 1995), FSH (Pareek et al. 2007; O’Shaughnessy 2014), and testosterone (Russell et al. 1987; Ruwanpura et al. 2010) are survival and differentiation factors of male germ cells. In our study, the concentration of LH and FSH did not change after administering pCA, indicating that the increased number of TUNEL-positive germ cells in pCA-treated rats is related to the low levels of testosterone. The mechanism that links pCA–testosterone–germ cell positive to markers of apoptosis is unknown. However, several factors suggest an oxidative damage mechanism: (1) testosterone depresses resistance to oxidative stress (Jeremy et al. 2021; Mohammadzadeh et al. 2021) and (2) oxidative damage contributes to amphetamine-induced toxicity, which leads to the death of neural and pulmonary cells (Andreazza et al. 2008; Chen et al. 2017). Thus, the DNA damage observed as an increase in germ cells positive to TUNEL could indirectly effect oxidant stress induced by pCA and the dysregulation of testosterone secretion.

Herein, we observed multinucleated giant cells as part of seminiferous tubule’s degeneration. The multinucleated giant cells were not TUNEL positive, indicating that their formation was due to dysregulation of the clonal differentiation process. This result was due to unknown reason; however, there is evidence that suggests a role of serotonergic communication in the maintenance of seminiferous epithelium: (1) the receptors 5-HT2A, 5-HT1A (Frungieri et al. 2002), and 5-HT7 (unpublished results) are present in the testis; (2) the protein Elmo1 plays a role in the clearance of apoptotic germ cells by Sertoli cells and Elmo1-deficient mice have a high number of multinucleated giant cells (Elliott et al. 2010), (3) substances such as 5-methoxy-N,N-dimethyltryptamine (5-MeO-DMT) upregulate Elmo1 through the 5-HT2A receptor (Dakic et al. 2017); thus, disruption of serotonergic communication, induced by pCA, could lead to the production of multinucleated giant cells in the seminiferous epithelium.

In general, the number of germ cells increases with age (Singh et al. 2015), which is consistent with the increase of testis weight observed in the control group animals. The intraperitoneal administration of pCA impairs testosterone secretion and induces apoptosis markers in seminiferous epithelium. Accordingly, the increase of germ cells positive to apoptotic markers could explain the low testicular weight observed after the treatment with pCA. The loss of cellularity leads

### Table 1. Effects of the treatment on morphology of seminiferous tubules, arithmetic mean ± s.e.m.

| Treatment | Area of seminiferous tubule ($\mu m^2$) | Area of lumen ($\mu m^2$) | Area of seminiferous epithelium ($\mu m^2$) | Circularity of seminiferous tubule |
|-----------|----------------------------------------|---------------------------|------------------------------------------|----------------------------------|
| AC        | 61181 ± 5650                           | 2423 ± 971                | 59089 ± 7730                            | 0.92 ± 0.03                      |
| VH        | 63721 ± 6774                           | 2071 ± 1169               | 61650 ± 6650                            | 0.91 ± 0.02                      |
| pCA       | 31340 ± 4346*                          | 6170 ± 2426*              | 25150 ± 4898*                           | 0.81 ± 0.06*                     |

$N = 6$ rats per group. *$p < 0.05$ versus VH and AC. AC: absolute control; VH: vehicle group; pCA: para-chloroamphetamine group. At least, 600 seminiferous tubules per treatment were analyzed.

### Table 2. Effects of the treatment on TUNEL and active caspase-7 labeling, arithmetic mean ± s.e.m.

| Treatment | Seminiferous tubules with TUNEL-positive germ cells (%) | Number of TUNEL-positive germ cells per seminiferous tubule | Seminiferous tubules with caspase-7-positive germ cells (%) | Number of caspase-7-positive germ cells per seminiferous tubule |
|-----------|--------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|
| AC        | 24 ± 1.16                                              | 0.51 ± 0.05                                              | 1.33 ± 0.88                                              | 2.00 ± 1.08                                              |
| VH        | 31 ± 2.12                                              | 0.83 ± 0.23                                              | 2.50 ± 0.50                                              | 2.50 ± 0.32                                              |
| pCA       | 58 ± 3.66*                                             | 3.25 ± 0.20*                                             | 5.33 ± 2.40*                                             | 9.00 ± 0.69*                                             |

$N = 6$ rats per group. *$p < 0.05$ versus VH and AC. AC: absolute control; VH: vehicle group; pCA: para-chloroamphetamine group. At least, 600 seminiferous tubules per treatment were analyzed.
to changes in seminiferous tubule morphology, such as a reduction in circularity and area.

Finally, the reduction in testosterone’s circulating concentrations allowed us to understand temporality’s role in evaluating the effects induced by pCA. During stages sensitive to the establishment of spermatogenesis, exposure to derivatives of amphetamine has a negative impact on the structure and physiology of the seminiferous epithelium. Whether pCA induced effects on fertility, once the exposed animals reach the adult stage is unknown emphasizing the need to investigate the fertility of the rats, and the possible effects on their descendants.

Materials and methods

Animals

Male rats of the CII-ZV strain, from our breeding stock, were maintained under controlled light conditions (lights on from 05:00 to 19:00), with free access to food (Nutricubos, Purina S.A., Mexico) and tap water.

Experimental design

The first experiment intended to analyze hormonal and neurotransmitter effects after pCA treatment. Male rats, of 30 days-of-age, were randomly assigned to one of the following experimental groups: untreated (absolute control, AC), injected intraperitoneally with saline solution (0.9%) (vehicle, VH) or with 10 mg/kg of body weight of pCA (Sigma-Aldrich, St. Louis, MO). The experimental groups were of 10 rats each. The dose of pCA administered was the effective dose that decreases the concentrations of serotonin and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the brain (Sanders-Bush et al. 1972a). Groups of animals were euthanized at 31, 32, 33, 34, or 35 days-of-age, then concentrations of serotonin and 5-HIAA in the anterior and medium hypothalamus, serum concentrations of progesterone, testosterone, and gonadotrophins were measured in each experimental group.

For the second experiment, we evaluated the effects of pCA on the seminiferous epithelium. Groups of six rats, of 30 days-of-age, received the same treatment as specified above and were euthanized at 35 days-of-age.

Autopsy procedure

The animals were euthanized in a CO2 chamber. The brain was quickly removed, placed in cold saline solution, and frozen in liquid nitrogen. After careful removal of the nerves and optic chiasm, the anterior and medium hypothalamus, and the hypophysis were dissected following the parameters described previously (Paxinos and Watson 2007). The anterior hypothalamus (Bregma 20.8 to Bregma 21.8) included the lateral and median preoptic nuclei, the suprachiasmatic, the paraventricular, the periventricular, and the stria terminal preoptic areas. The medial hypothalamus (Bregma 22.3 to Bregma 23.2) included the median eminence and arcuate nucleus. Both hypothalamic regions and hypophysis were stored at −70°C until the concentration of serotonin and 5-HIAA were measured.

Blood was collected from the trunk of the animals, allowed to clot, and subsequently centrifuged at 1000 g for 15 min. The serum supernatant was collected and stored at −20°C. The testes, the epididymis, the prostate, and the seminal vesicles were dissected and individually weighed on a precision balance.

Measurement of serotonin and 5-HIAA

The concentration of serotonin and 5-HIAA were measured using the previously described methodology (Monroy et al. 2003). Briefly, the tissue samples were weighed and homogenized in 0.1 N perchloric acid (150 μL for the pituitary, and 300 μL for the anterior and median hypothalamus). The homogenates were centrifuged at 12,500 rpm for 30 min at −4°C. The supernatant was filtered using 0.2 μm regenerated cellulose filters. One hundred microliters of the extract were injected into the HPLC system (Perkin-Elmer Co., Waltham, MA Model LC-250). The concentrations of serotonin and 5-HIAA were quantified electrochemically, using an LC-4C amperometric detector and an LC-4A glassy carbon transducer cell at a potential of 850 mV. The results are expressed as ng of 5-HT/mg of tissue.

Gonadotropin and sexual hormone determination

Serum concentrations of progesterone and testosterone were measured by specific radioimmunoassay (RIA), using kits purchased from Diagnostic Products (Los Angeles, CA). The intra- and inter-assay coefficients of variation were 5.3 and 9.87% for progesterone and 4.3 and 7.8% for testosterone, respectively. The detection limits for progesterone and testosterone were 0.09 and 0.005 ng/ml, respectively. For LH and FSH, we used RIA with the double antibody technique using reagents and protocol supplied by the National Hormone and Pituitary Programs (Baltimore, MD).
The values were expressed in ng/ml in terms of the reference preparations of NIH LH-RP2 and FSH-RP2. The intra- and inter-assay coefficients of variation were 7.91 and 5.74% for LH and 9.3 and 6.82% for FSH. The detection limits for LH and FSH were 0.05 and 0.01 ng/ml, respectively.

**Testicular tissue processing**

To evaluate the effects of pCA on the seminiferous epithelium, the rats were anesthetized with sodium pentobarbital (40 mg/kg of body weight, ip), intracardially perfused with saline solution 0.9%, and fixed with paraformaldehyde 4% in phosphate-buffered saline (pH = 7.4) (Sigma-Aldrich, St. Louis, MO, USA). After perfusion, the testes were removed and immersed in the same fixative for 24 h at 4°C, followed by 70% ethanol during 24 hours and finally dehydrated through a graded series of ethanol concentrations, clarified in xylene, and embedded in paraffin wax (Leica Biosystems, Nussloch, Germany). Serial 5 µm thick sections of the right testis were taken every 100 µm and mounted on glass slides coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO).

**TUNEL and active caspase-7 analysis**

For the detection of DNA fragmentation, end-labeling was performed with the aid of an apoptosis kit according to the manufacturer’s instructions (Roche, Boehringer Mannheim Corp., Indianapolis, IN). Briefly, sections were deparaffinized, hydrated, and treated with 20 mg/ml proteinase K (Roche) for 30 min at 37°C, in a humidified chamber. The endogenous peroxidase was blocked for 20 min with block solution (Roche Diagnostics, Indianapolis, IN). The tissue was treated with permeabilization solution (0.1% Triton in 0.1% sodium citrate) for 2 min; then incubated with label solution (terminal deoxynucleotidyl transferase (TdT) enzyme and biotinylated 16-dUTP) for 60 min. After each step, the slides were rinsed twice in PBS for five minutes each. Finally, the tissue was incubated with converter-POD (anti-fluorescein conjugated with horseradish peroxidase; 30 min at 37°C), counterstained with hematoxylin, and mounted with Permount (Fisher Scientific, Fair Lawn, NJ). Testicular cells with dark-brown-staining were considered caspase-7 positive. A negative control was made following the same steps previously described but PBS was used instead of the active caspase-7 antibody. At least, 100 seminiferous tubules per rat were randomly chosen and analyzed for the presence of caspase-7-positive germ cells. Mean percentages of seminiferous tubules with germ cells TUNEL-positive per seminiferous tubule, are presented.

For immunohistochemical detection of active caspase-7, the sections were dehydrated and endogenous peroxidase was blocked with 3% H2O2. Non-specific binding was blocked by incubating the slides for 1 h at room temperature with 10% normal goat serum. The sections were incubated overnight with 40 µl of active anti-caspase-7 polyclonal antibody (Dilution: 1:100, Abcam, Cambridge, UK) in a wet chamber. After each step, the sections were washed with PBS for five minutes. The secondary antibody was incubated with horseradish peroxidase-conjugated to biotinylated anti-rabbit IgG from ABC Elite Vectastain (Vector Laboratories, Inc. Burlingame, CA) for 45 min. The sections were visualized by diaminobenzidine (DAB) (Vector), counterstained with hematoxylin, and mounted with Permount (Fisher Scientific, Fair Lawn, NJ). Testicular cells with dark-brown-staining were considered caspase-7 positive. A negative control was made following the same steps previously described but PBS was used instead of the active caspase-7 antibody. At least, 100 seminiferous tubules per rat were randomly chosen and analyzed for the presence of caspase-7-positive germ cells. Mean percentages of seminiferous tubules with germ cells active caspase-7-positive and the mean number of germ cells caspase-7-positive per seminiferous tubule are presented.

**Morphometric analysis of seminiferous tubules**

Seminiferous tubules were observed using an Otiphot-2 microscope (Nikon Instruments, Melville, NY) at 100× or 400× total magnification. Micrographs were taken with an iPhone (model A1723) coupled to a 3D-printed custom microscope adapter.
circularity and area. The area of the seminiferous tissue was calculated as area of the seminiferous tubule minus area of the lumen. At least, 100 seminiferous tubules per rat (600 seminiferous tubules per treatment) were randomly chosen and analyzed for morphometry.

**Statistical analysis**

The normality of the data was confirmed for each experiment by checking residuals. For the first experiment, two multivariate analyses of variance (MANOVA), using treatment and day of euthanasia as dependent variables, were conducted. The first MANOVA grouped body and sexual organ weights, the second MANOVA included concentrations of serotonin, gonadotrophins, and sex steroids. Variance not explained (lambda, Λ) and explained (eta-square, η²) by each MANOVA are provided. When MANOVA was significant, two-way ANOVA was conducted, considering chemical treatment as the factor and day of euthanasia as the block. Linear contrasts of means (orthogonal contrast) were performed for each two-way ANOVA model (H0: μAC = μVH and H0: μPCA = μAC + μVH/2). For the second experiment, morphometric parameters were compared by one-way ANOVA followed by linear contrasts as specified above. Percentages of germ cells positive to TUNEL or caspase-7 were compared using the Chi-square test. The results were considered significant when p < 0.05. Results are presented as mean ± s.e.m. All analyses were performed with the R software version 2.13.1 (R Core Team 2017) running on a MacBook with Mac OSX version 10.6.2.

**Ethical approval**

All experiments were performed following the guidelines established by the Mexican Law of Animal Protection Guidelines Treatment. The Committee of the Facultad de Estudios Superiores Zaragoza, UNAM, approved the experimental protocols (Letter 04/22/2021). All efforts were made to minimize animal suffering.

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**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Authors’ contributions**

Conceived and designed the experiments: MEA, AA. Performed the experiments: CR, MF, EG, JP. Analyzed the data: CR, MEA, and AA Contributed reagents analysis tools: MC. Wrote the manuscript: MEA, AA, and CR. Final edit of the paper: MEA, and AA.

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