

PREPUBERAL STRESS AND OBESITY: EFFECTS ON SERUM CORTICOSTERONE, PROLACTIN, TESTOSTERONE AND PRECANCEROUS PROSTATE LESIONS IN ADULT RATS

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Stress during puberty and obesity can represent conditions that facilitate the long-term development of diseases, especially for stress-related disorders that depend on neuroendocrine and immune responses. The prostate is prone to diseases that result from neuroendocrine or immune challenges, such as cancer. **Aim:** In the present study, we assessed the long-term effects of an acute pubertal stressor (immune-challenge) or obesity on the development of precancerous lesions in rats. **Materials and Methods:** Pubertal male rats received a single injection of lipopolysaccharide (LPS) or saline during puberty (5 weeks of age). In adulthood (8 weeks old), subgroups of males were fed with hypercaloric liquid diet to induce obesity. This resulted in a total of six subgroups: (1) intact-non obese, (2) intact-obese, (3) saline-non obese, (4) saline-obese, (5) LPS-non obese, and (6) LPS-obese. At 16 weeks of age the rats were sacrificed for prostate histology (hematoxylin and eosin stain) and hormone analysis (testosterone, corticosterone and prolactin). **Results:** As compared to intact-non obese rats, males treated with LPS and those with obesity expressed histological alterations in both the dorsolateral and ventral portions of the prostate. Only prolactin was altered in LPS-treated males, whereas corticosterone was altered in LPS-obese rats. **Conclusions:** These results indicate that pubertal exposure to an immune challenge or obesity facilitate the development of prostatic lesions in adult male rats. We discuss the role of hormones in the development of precancerous lesions. **Key Words:** prostate, cancer, stress, puberty, obesity, lipopolysaccharide.

The prostate is an exocrine gland whose secretion facilitates fertility (e.g. sperm transport, and spermatozoa activation/survival) [1–3]. As any other gland, it can be affected by processes of inflammation (prostatitis), progressive enlargement (benign hyperplasia, BPH), or cancer (CaP). The development of CaP is complex, and depends on multiple risk factors that can be inherited and/or environmental. For instance, precancerous lesions (e.g. epithelium dysplasia, anisocytosis, anisokaryosis, nucleus apolarity, etc.) can be found in individuals with a previous history of chronic prostatitis due to infections [4]. However, the development of CaP is mainly linked to chronic high levels of hormones that facilitate cell proliferation like testosterone [5, 6] or prolactin [7–9]; and it is also linked to factors such as older age [10], african-ancestry [11], and smoking [12]. Interestingly, other factors such as the consumption of high-fat products [13], obesity [14] and stress [15] appear to play an important role in the risk for developing CaP or precancerous prostate lesions, which represent the anteroom for CaP.

The link between diet, obesity and stress in the development of CaP is not completely understood, but some studies indicate that these conditions may result in impairment of the neuroendocrine system and chronic sub-clinical inflammation [16]. For example, obese individuals express up to 4-fold higher levels of steroid hormones like

androstenedione and estrogen, but also insulin, cortisol, prolactin and leptin [17–19], which can facilitate tumoral growth by enhancing cell proliferation, differentiation and survival. Likewise, chronic stress in adulthood (i.e. restrain) increases the transcript levels of several genes associated with cellular proliferation in the prostate [20], and acute stress during critical periods of development (i.e. puberty) results in precancerous prostate lesions in adulthood [15].

Herrera-Covarrubias *et al.* (2017) used 5-week old prepubertal male rats that received one single injection with the bacterial endotoxin lipopolysaccharide (LPS) to induce stress and immune responses [21–23]. Thus, treatment with LPS triggers an immune response that results in the production of cytokines, cyclooxygenase 2 (COX-2), and prostaglandins (prostaglandin E₂, PGE₂) among other molecules [23], which also have an effect on the brain, and activate the hypothalamic-pituitary-adrenal (HPA) axis. Treatment with LPS at 6 weeks of age (puberty) results in permanent neuroendocrine alterations, but it does not occur if LPS is injected during the postnatal weeks 3, 7, 8 or 10 [24], indicating that long-term effects of LPS treatment occur exclusively when it is experienced during the pubertal stress sensitive period (5–6 weeks old). Then, at 12 weeks of age the prostates of those rats were analyzed for histological abnormalities. Males from the LPS group expressed precancerous lesions in both the dorsolateral and ventral portions of the prostate (e.g. epithelium dysplasia, anisocytosis, anisokaryosis, nucleus apolarity), indicating that one episode of acute stress right before puberty can induce long-term effects on the prostate's health [15]. Thus, given that the link between obesity and acute prepubertal stress in the development of CaP is not well understood, the present study was designed to assess their single and combined effects in rats. We hypothesized that combination of the

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Abbreviations used: ANOVA – analysis of variance; BPH – benign prostatic hyperplasia; CaP – prostate cancer; COX2 – cyclooxygenase 2; DLP – dorsolateral prostate; ELISA – enzyme-linked immunosorbent assay; H & E – hematoxylin and eosin; HPA – hypothalamic-pituitary-adrenal; IL – interleukin; IFN – interferon; i.p. – intraperitoneal; LPS – lipopolysaccharide; NF-κB – nuclear factor kappa light chain enhancer of activated B cells; PGE₂ – prostaglandin E₂; TNF – tumor necrosis factor; VP – ventral prostate.

two risk factors would alter the serum levels of testosterone, prolactin and corticosterone, which would result in more severe precancerous lesions or CaP.

MATERIALS AND METHODS

Animals. Thirty six Wistar male rats (*Rattus norvegicus albinus*) were purchased and shipped at 4 weeks of age from a certified laboratory animal supplier in Mexico (Circulo ADN®). They were housed in groups of five rats in large plexiglas cages (50 × 30 × 20 cm) and kept in a colony room at the Centro de Investigaciones Cerebrales, Universidad Veracruzana, Mexico, in a 12–12 h reverse light-dark cycle (lights off at 8:00 hrs). Water and commercial rat chow (Rismart®) were always provided *ad libitum*. All the experimental procedures were carried out according to the Official Mexican Norm for use and care of laboratory animals (NOM-062-ZOO-1999).

Groups and treatments. The rats were randomly assigned to one of the following three groups: intact, saline-treated, or LPS-treated. Each group was divided into subgroups of non-obese or obese rats, resulting in six subgroups: 1) intact-non obese (n = 6), 2) intact-obese (n = 6), 3) saline-non obese (n = 6), 4) saline-obese (n = 6), 5) LPS-non obese (n = 6), and 6) LPS-obese (n = 6). Table 1 indicates the treatment received by each group and the age (weeks) at the time of treatment. At 5 weeks of age (puberty), rats from subgroups 5 and 6 received one intraperitoneal (i.p.) injection of LPS (LPS from *E. coli*, Sigma-Aldrich Catalogue L3755) at a dose of 1.5 mg/kg in a volume of 1 ml of sterile saline (0.9%). Subgroups 3 and 4 received exclusively a saline injection (i.p.) and subgroups 1 and 2 received no injections. Rats were monitored at 2, 4, 8, 24 and 48 h following the injection, and we scored the presence of five sickness symptoms (ptosis, piloerection, lethargy, huddling, xyphosis) as previously reported [15]. At each time-point, rats were given a score ranging from 0 to 5, depending on the number of symptoms observed (Fig. 1). During the following three weeks, the rats were handled daily, and their general health was monitored. Starting at 8 weeks of age, subgroups 2, 4 and 6 received additional hyper caloric liquid diet consisting of 97 kcal/100 ml, 15% protein, 30% fat, 54% carbohydrates (ensure advance®). Sufficient liquid hyper caloric diet was provided *ad libitum* (at least 100 ml *per rat/day*) in order to produce a minimum difference of 15% of body weight between non-obese and obese rats in every group. Such weight difference was reached by week 16 of age (Fig. 2, Table 1).

Prostate samples and histology. At 16 weeks of age the rats were deeply anesthetised with sodium pentobarbital (65 mg/kg i.p.). Then, 3 ml of blood were obtained by cardiac puncture for analysis of testosterone, corticosterone and prolactin (see *Hormone measurements* for further details). Anesthetised rats were then sacrificed with an overdose of sodium pentobarbital (120 mg/kg i.p.). An abdominal incision was performed and the accessory sexual organs were carefully removed and placed into a container

Table 1. Groups, age and treatments

Subgroup	Treatment		
	5 week-old, single i.p. injection	8–16 week-old, diet	16 week-old
(1) Intact-non obese	Nothing	Regular chow	Hormones + histopathology
(2) Intact-obese	Nothing	Regular chow + hypercaloric	Hormones + histopathology
(3) Saline-non obese	Saline	Regular chow	Hormones + histopathology
(4) Saline-obese	Saline	Regular chow + hypercaloric	Hormones + histopathology
(5) LPS-non-obese	LPS	Regular chow	Hormones + histopathology
(6) LPS-obese	LPS	Regular chow + hypercaloric	Hormones + histopathology

with 0.9% saline solution. The prostate was identified under a dissecting microscope (MEJI, EMZ-TR®) and it was further divided into ventral (VP) and dorsolateral prostate (DLP). As in our previous studies [7, 15, 25] the VP and DLP were soaked in 10% formalin for 24 h, then dehydrated in 70% and 80% alcohol (1 h each), and 95% (3×2 h each), and 100% ethanol overnight, plus two more changes (1 h each), the following day. Then xylene (3×1 h each), always in constant shaking. Tissue was embedded in paraffin wax 2×2 h each), sliced

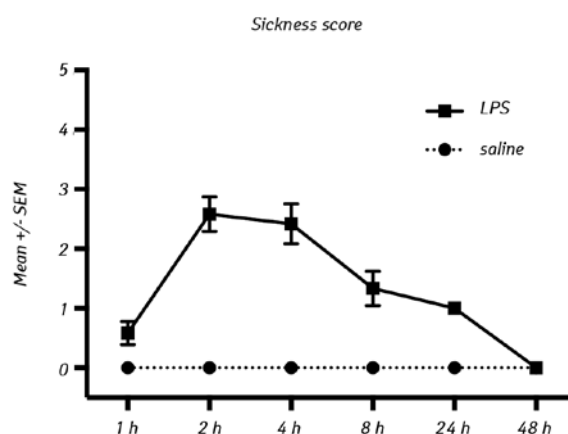


Fig. 1. Sickness score of male rats injected at 5 weeks of age (prepuberal) with LPS from *E. coli*. Only LPS-treated males expressed symptoms, indicative of a stress response

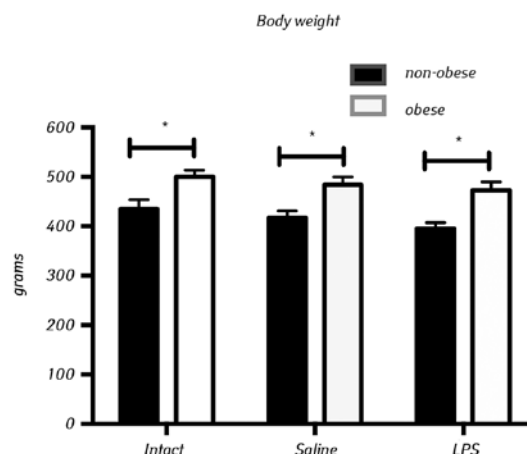


Fig. 2. Body weight of male rats at 16 weeks of age. Black bars represent subgroups fed exclusively with regular rodent chow. White bars represent subgroups that in addition to rat chow received liquid hyper-caloric diet starting at 8 weeks of age

(5 µm thick) with a microtome (RM 2125RT Leica®), mounted on slides in a bath at 52 °C (containing pork skin-based gelatin 2.5 mg/100 ml) and then processed for hematoxylin and eosin (H & E) dye technique as follows: 1 h at 57 °C, deparaffinization in xylene (3×5 min each), rehydrated in alcohol/xylene (1:1) 5 min, ethanol 96% 3 min, hematoxylin (10 min), water (30 s), acid alcohol (quick immersion), water (10 s), lithium carbonate (30 s), water (10 s), eosin (4 quick immersions). Dehydration in ethanol 96% (3 min), ethanol 100% (2 min), ethanol/xylene 1:1 (2 min), and xylene (5 min). Then, the slides were coverslipped with Permount, air dried, and observed under a light microscope (Olympus Ax70). Photomicrographs were taken at 40× and analyzed by the same experimenters. As formerly reported [7, 25], we assessed prostate histology from normal (expected) to abnormal (non-expected) by taking into consideration 12 histological features observed in the intact non-obese subgroup (Table 2).

Table 2. Characterization of both normal (expected) and abnormal (non-expected) histology in the prostate of adult rats. Normal features were inferred from the number of cases observed in group 1 (intact-non obese) of the present study

Histological feature	Region	Normal (expected)	Abnormal (non-expected)
Epithelium form	DLP	Cubic	Metaplasia,
	VP	columnar	dysplasia
Epithelium size	DLP	Even	Anisocytosis
	VP		
Epithelium papillae	DLP	Scarce	Plenty
	VP		
Interstice space	DLP	Even	Compressed
	VP		
Interstice content	DLP	Collagen	Mononuclear cells
	VP		
Nucleus size	DLP	Even	Anisokariosis
	VP		
Nucleus location	DLP	Basal cell	Non-polar
	VP	polarity	
Nucleus-cytoplasm ratio	DLP	1:3	< 1:3
	VP		
Myoepithelium	DLP	Euplasia	Proplasia
	VP		
Pattern (at 4×)	DLP	Tubular	Cribiform
	VP		
Lumen content	DLP	Amorphous	Granular
	VP		
Chromatin	DLP	Heterochromatin	Euchromatin
	VP		

Hormones measurement. Following deep anesthesia, 3 ml of blood were obtained by cardiac puncture and the concentrations of testosterone, corticosterone and prolactin in serum were measured. Blood was collected in vacutainer tubes containing no anticoagulant and incubated in upright position at room temperature for 30 min to allow clotting. Tubes were centrifuged for 15 min at 1000 rpm. Supernatant was aspirated at room temperature and serum was kept in 500 µl aliquots and frozen at −20 °C for a few days until processing. Hormones levels were quantified using Enzyme-Linked Immuno Sorbent Assay (ELISA) and commercial kits for testosterone (ALPCO 11-TESHU-E01), corticosterone (ALPCO 55-CORMS-E01) and prolactin (ALPCO 55-PRLRT-E01). The procedure was carried out as instructed by the supplier. The assays were read

in an IMARK microplate reader with the software microplate manager from Bio-Rad.

Variables and statistical analysis. Sickness score. We examined the intensity (0–5) and duration (0–48 h) of sickness after treatment with LPS during puberty. **Histology:** We examined 12 histological features in each male in adulthood (see Table 2 and section *Prostate samples and histology*). One-tailed Fisher exact tests were performed to calculate significant differences between the number of normal (intact non-obese) vs abnormal cases (other groups). **Body weight** (grams) and **Hormones** (ng/ml) were analyzed with a two-way (group X body condition) analysis of variance (ANOVA), followed by a Fisher LSD *post hoc* test to compare individual differences. All statistical analyses were performed using GraphPad Prism version 6.00 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com and the alpha level was set at $p < 0.05$.

RESULTS

Sickness score during (puberty) and obesity (adulthood). Of the 12 rats that received LPS during puberty (5 weeks old) all expressed sickness symptoms, which indicates that rats underwent a stress response. The most common symptom were lethargy (100%) and huddling (100%), followed by ptosis (75%), piloerection (33%) and xyphosis (0%). None of the males from the saline group expressed symptoms after injection. Fig. 1 depicts the sickness score, indicating that a maximum peak response occurred 2 h after injection and lasted for less than 48 h. With regard to the induction of obesity, the results indicated that rats that received liquid hypercaloric diet (obese subgroups) increased at least 15% or more body weight than the non-obese subgroups (Fig. 2). Accordingly, puberal stress and obesity in adulthood were properly achieved.

Prostate histology in adulthood. The analysis of the prostate showed that both experimental conditions (puberal stress or obesity) resulted in abnormal prostatic histology in adulthood. Fig. 3–6 depict some of the effects on the DLP and VP portions, respectively. For example, in the DLP both factors, obesity and LPS, induced more cases of epithelium dysplasia (Fig. 3, a), anisocytosis (Fig. 3, b), anisokariosis (Fig. 3, d), and non-basal polarity (Fig. 3, e). LPS alone (but not obesity) induced presence of mononuclear cells (Fig. 3, c), and also abnormal nucleus-cytoplasm ratio in both obese or non-obese rats (Fig. 3, f). Both obesity and LPS caused proplastic myoepithelium and compressed interstice space, but did not induce the presence of papillae, pattern, lumen content, or chromatin (Fig. 4). With regard to the VP, obesity and LPS also induced histological changes, however, the probability of reaching significant differences depended on the number of normal cases observed in the intact non-obese group. For instance, there were many cases of dysplasia and metaplasia in the groups saline-obese (5/6), LPS-non obese (3/6) and LPS-obese (5/6); however, those frequencies were not significantly different to intact-non obese rats that expressed 2/6 cases

of metaplasia (Fig. 5, a). Obesity alone (but not LPS) caused anisocytosis (Fig. 5, b) and non-basal nucleus polarity (Fig. 5, e). Both conditions induced presence of mononuclear cells (Fig. 5, c), anisokaryosis (Fig. 5, d) and also abnormal nucleus-cytoplasm ratio (Fig. 5, f). Both conditions caused compressed interstice and propasia in myoepithelium. Only LPS caused the presence of papillae, and granular lumen content (Fig. 6).

Serum levels of testosterone, corticosterone and prolactin. With regard to testosterone, the ANOVA failed to detect significant differences between groups $F(2,30) = 0.13$, $p > 0.05$ (Fig. 7, a), indicating that neither obesity nor LPS treatment affected the adult baseline serum levels. However, with regard

to corticosterone there was a significant interaction between obesity and puberal stress $F(2,30) = 5.05$, $p = 0.01$ (Fig. 7, b). The *post hoc* test indicated that LPS-obese rats expressed significantly higher corticosterone levels (mean = 1130 ng/ml) than LPS-non obese (mean = 778 ng/ml). This indicates that obesity is a significant cause of stress in adulthood (as observed via corticosterone levels), particularly in rats that underwent prepuberal stress treated with LPS. With regard to prolactin there was a main effect of group $F(2,30) = 3.1$, $p = 0.05$ (Fig. 7, c). The *post hoc* analysis indicated that rats from the LPS group (mean = 109 ng/ml) displayed higher prolactin levels than rats from the intact group (mean = 80 ng/ml). This

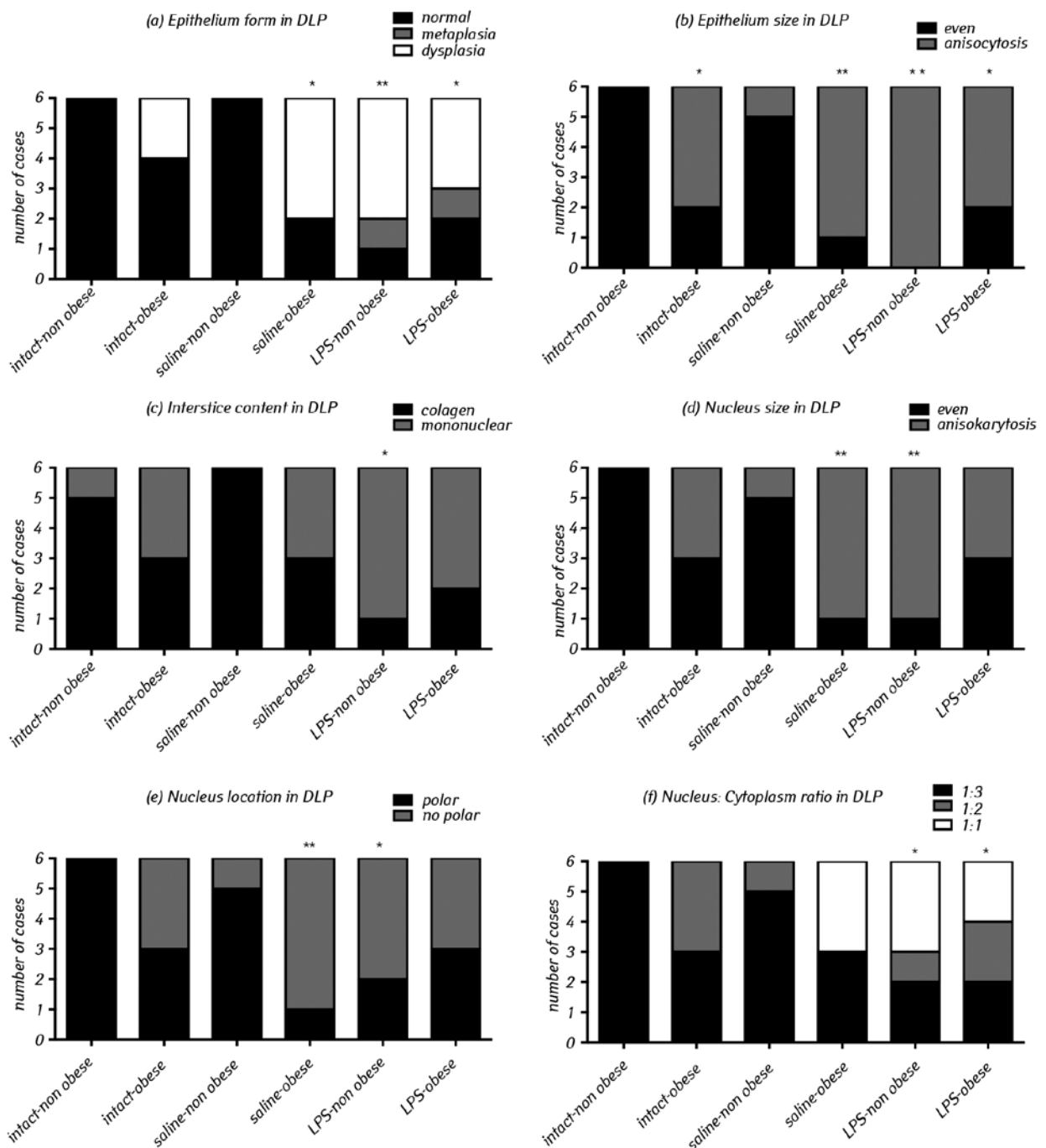


Fig. 3. Histological features in the DLP of rats from different groups. Normal (expected) features are indicated in black, whereas abnormal features are shown in gray and white. * $p < 0.05$ and ** $p < 0.01$ compared to intact-non obese subgroup

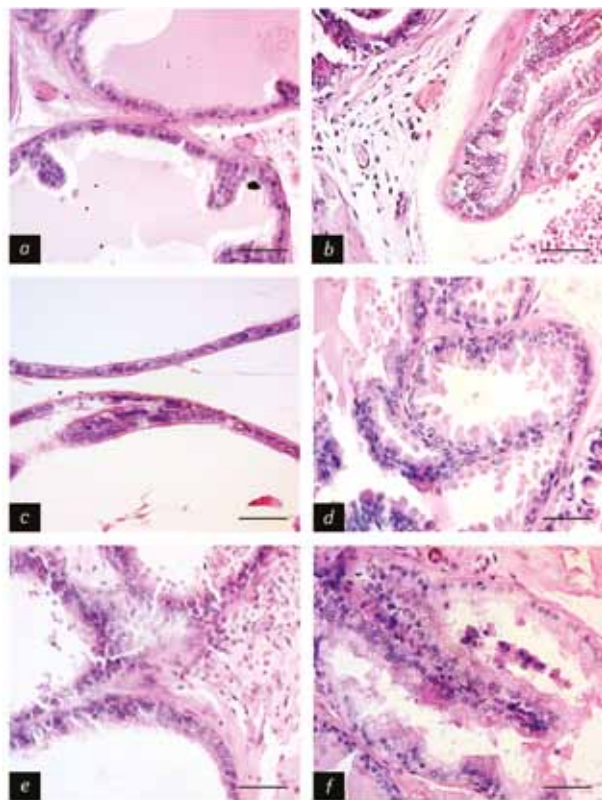


Fig. 4. Photomicrographs (40×) of the DLP of rats from different groups stained with H & E. Normal (expected) features are indicated in (a) intact-non obese rats and (c) saline-non obese, whereas abnormal features are shown in (b) intact-obese, (d) saline-obese, (e) LPS-non obese and (f) LPS-obese. Bar = 50 μ m

indicates that regardless of obesity in adulthood, rats that underwent prepuberal stress expressed higher prolactin basal levels in adulthood.

DISCUSSION

The present study was designed to assess the effects of prepuberal stress and adult obesity on the development of prostatic lesions and on the baseline serum levels of testosterone, corticosterone and prolactin. Our results showed that both risk factors, either alone or combined, caused abnormal histological features in the DLP and VP in adult male rats. Only prepuberal stress (regardless of body weight) resulted in higher baseline levels of prolactin in adulthood. In addition, the interaction between prepuberal stress and obesity resulted in higher baseline levels of corticosterone, but none of the two factors resulted in significant increases in the levels of testosterone.

As expected, rats from the intact-non obese subgroup showed no prostatic alterations in the DLP. However, rats with prepuberal stress or rats with obesity, expressed more cases of epithelium dysplasia, anisocytosis, compressed interstice, anisokariosis, proplastic myoepithelium, granular content and abnormal nucleus-cytoplasm ratio (see Fig. 3, 4). In the VP, however, some intact-non obese rats expressed metaplasia (2/6), indicating that this type of abnormality may be expected in some control animals. Interestingly, in the VP more cases of metaplasia or dysplasia

were also observed in the groups saline-obese (5/6), LPS-non obese (3/3) or LPS-obese (5/6). In addition, the two factors caused presence of mononuclear cells, anisokariosis, abnormal nucleus-cytoplasm ratio, compressed interstice and proplastic myoepithelium, although only obesity resulted in more cases of anisocytosis and nucleus apolarity (see Fig. 5, 6). Accordingly, our results indicate that the DLP was relatively more affected by prepuberal stress (8/12 abnormal histological features) than by obesity (6/12), whereas the VP was relatively equally affected by obesity (7/12) or prepuberal stress (7/12).

Levels of hormones and the risk of precancerous prostate lesions. Puberty is considered a critical period for the long-term development of diseases, especially for those that depend on neuroendocrine and immune responses. During puberty the HPA axis is more responsive to stressors than in adulthood [26]. For example, pubertal male rats exposed to acute restraint stress (30 min) express longer responses of adrenocorticotrophic hormone and corticosterone [27]. One previous study from our laboratory showed that prepuberal LPS resulted in abnormal prostate histology in adult rats (12 week old) [15]. The lesions observed in that study were the same as those reported here. Likewise, that study showed that LPS had no effect on the baseline levels of serum testosterone in adulthood (mean = 7.3 ng/ml), nor on the levels of corticosterone (mean = 550 ng/ml). Herein, we found similar effects of LPS on serum testosterone (mean = 6.9 ng/ml), supporting the idea that LPS does not depend on testosterone modifications to affect the prostate. However, we cannot discard the possibility that LPS may affect the density of androgen receptors [28], as formerly reported in one report of human CaP that resulted from infections [29]. Androgen receptors gene can mutate or be amplified by LPS and can respond to lower or equal levels of androgens in adulthood [8, 30–32]. Therefore, it may be possible that LPS increases the density of androgen receptors, which would result in a more susceptible prostate under the effects of normal androgen levels.

However, our data showed that prepuberal LPS resulted in increased levels of corticosterone in adulthood, but only in obese rats (mean = 1130 ng/ml) as compared to non-obese rats (mean = 778 ng/ml). Accordingly, we argue that obesity in adulthood can be sufficiently challenging to maintain high baseline levels of corticosterone in rats that were sensitized by prepuberal stress. In our study, however, higher levels of corticosterone in LPS-obese rats did not result in more prostate lesions as compared to the LPS-non obese group. Thus, LPS-obese rats from our study probably were more stressed in adulthood, but their prostates were not necessarily more affected than prostates from the other groups. This suggests that not all prostate alterations are a consequence of chronic abnormal high HPA axis activity.

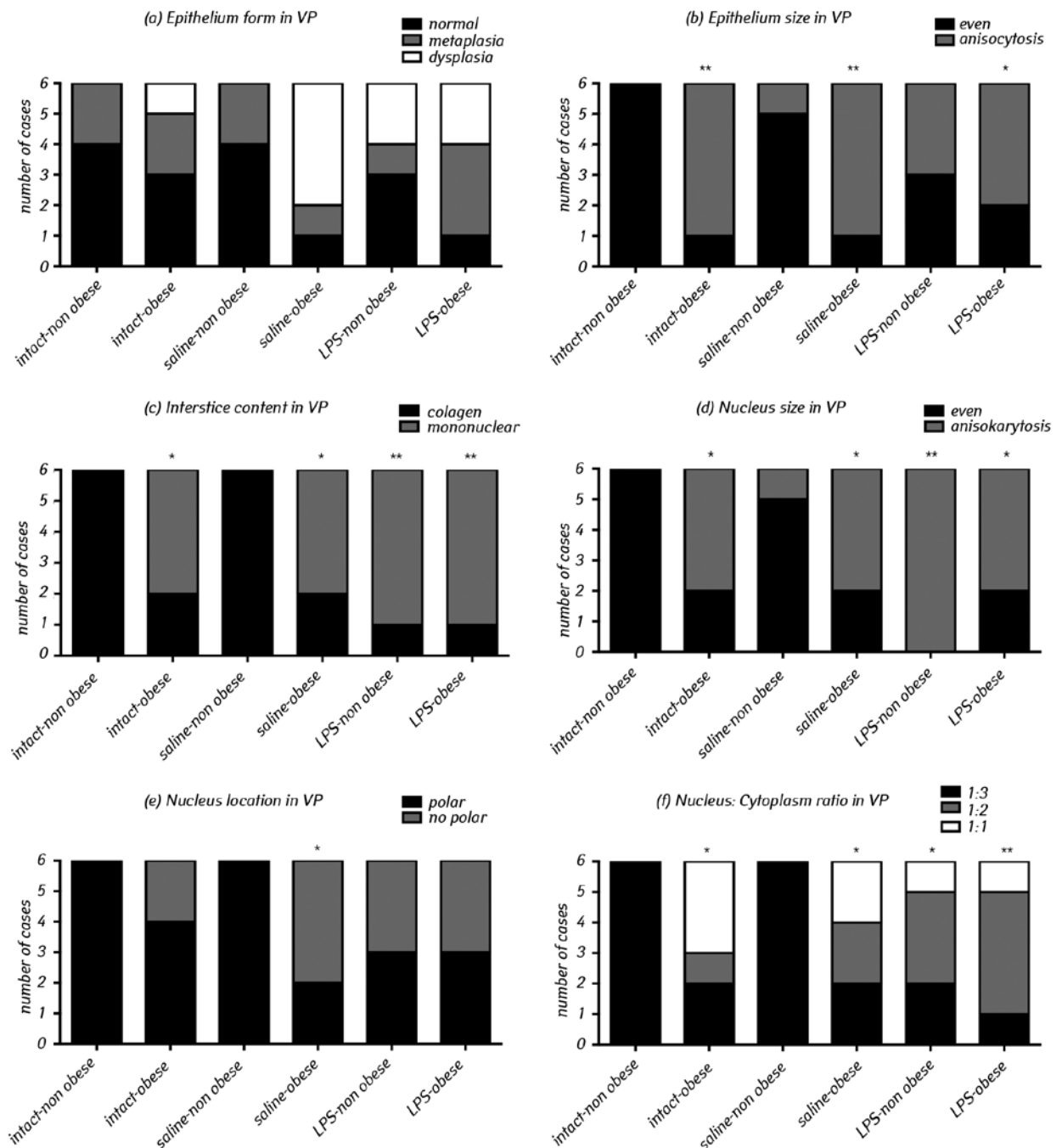


Fig. 5. Histological features in the VP of rats from different groups. Normal (expected) features are indicated in black, whereas abnormal features are shown in gray and white. * $p < 0.05$ and ** $p < 0.01$ compared to intact-non obese subgroup

Likewise, LPS-treated rats expressed higher baseline serum levels of prolactin in adulthood. Some studies have shown that serum levels of prolactin increase more in pubertal rats than in adults after chronic stress [33] and also in obese individuals [34]. Higher or longer exposure to prolactin is believed to result in enduring changes in hormone-sensitive organs. However, in the present study, more prolactin did not reflect worse prostatic lesions. In general, however, these data may suggest that different risk factors affect the prostate via different pathways. For example, prepubertal stress might impact the prostate via increased levels of prolactin, or *via* increased levels of corticosterone when combined with the challenge of obesity.

Indeed, a stress challenge on sensitized (prepuberally-stressed) individuals may trigger otherwise quiescent pathologies. Perhaps, obesity and different types of infections may trigger subclinical inflammation processes that result in histological lesions. In our study, LPS-treated or obese males displayed more mononuclear cells than the control group (see Fig. 3, c, 5, c) (indicating subclinical inflammation). One common cause of prostatitis are infections by *E. coli* and *Enterococcus spp.* [35, 36], which can result in epithelial proliferation and reactive hyperplasia, dysplasia and oxidative DNA damage [37, 38]. Other gram negative bacteria (LPS-holders) such as *Pseudomonas spp.*, *Proteus*

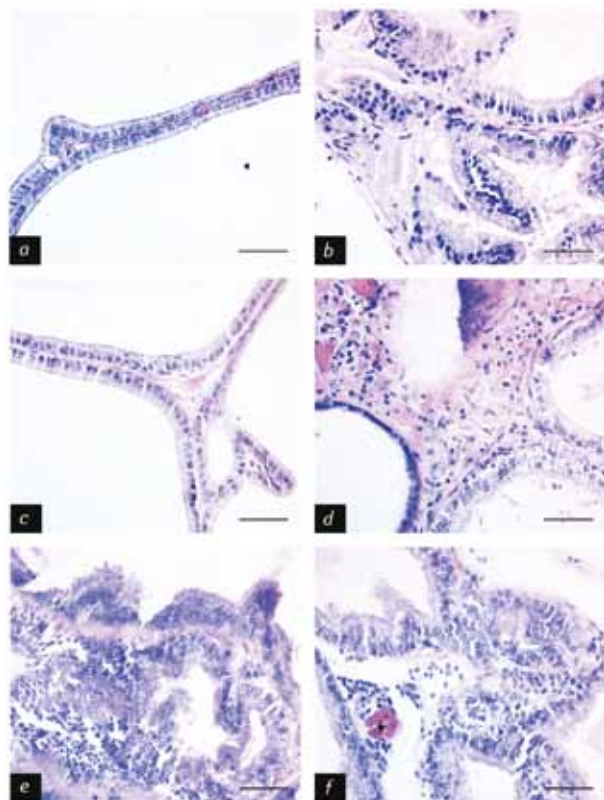


Fig. 6. Photomicrographs (40×) of the VP of rats from different groups stained with H & E. Normal (expected) features are indicated in (a) intact-non obese rats and (c) saline-non obese, whereas abnormal features are shown in (b) intact-obese, (d) saline-obese, (e) LPS-non obese and (f) LPS-obese. Bar = 50 μm

mirabilis, *Klebsiella spp.* and *Serratia spp.* and many other sexually transmitted organisms can also be considered a cause of prostatitis [4].

Infections, obesity and LPS can result in immune responses by altering the production of different cytokines such as interleukins IL-1b, IL-6, IL-10, IL-12 [23, 39], interferon gamma (INFγ), tumor necrosis factor alpha (TNFα) [23], and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [40], which can maintain inflammation, and can be considered a risk factor that mediates CaP development [41]. Interestingly, inflammation is very common within the adult human prostate [4], but different studies have found both positive [42, 43] and negative results [44] regarding its role in CaP.

CONCLUSIONS

Puberal stress or adult obesity result in precancerous histological alterations in the two prostatic portions (DLP, VP) in rats. Serum baseline levels of testosterone were not modified, and therefore unlikely to be the cause of LPS- or obesity-induced lesions. However, the levels of prolactin and corticosterone were enhanced by LPS or LPS-obesity, respectively, and therefore may be related to the histological lesions observed in those subgroups. We conclude that puberal stress and obesity participate in the development of prostatic precancerous lesions in adulthood.

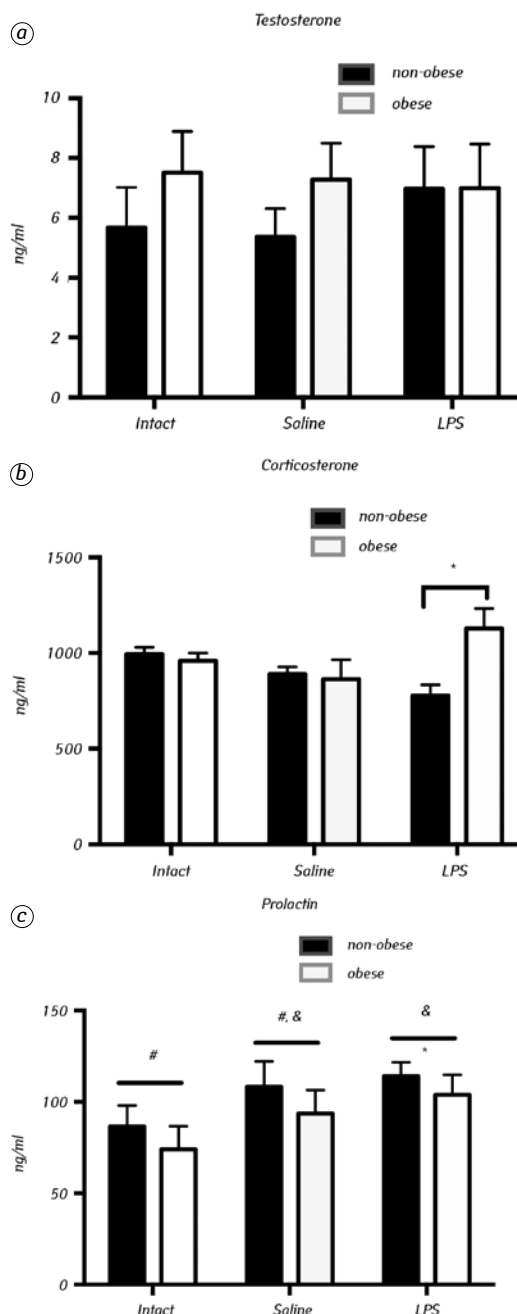


Fig. 7. Baseline serum levels of testosterone (a), corticosterone (b) and prolactin (c) in adult rats (16 weeks of age). The LPS group received LPS at 5 weeks of age. The saline groups received injectable-grade 0.9% sodium chloride saline. Intact rats did not receive any injection. Black bars represent non-obese subgroups, and white bars represent obese subgroups. * $p < 0.05$ between obese and non-obese rats. #, & $p < 0.05$ between groups

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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