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Low protein uptake during peripuberty impairs the testis, epididymis, and spermatozoa in pubertal and adult *Wistar* rats

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Original Article

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Abstract

Protein malnutrition during critical periods poses significant risks to reproductive health. Thus, this study aims to evaluate the immediate and delayed effects of a 30-day low-protein diet on the postnatal development of the male reproductive system. For so, male rats were fed a protein-deficient diet from postnatal day 30–60, followed by evaluations of testis, epididymis, and spermatozoa both at the end of the diet and after a 60-day recovery period. Testicular and epididymal weight was lowered in pubertal animals. Several histological alterations were found in the testis, such as acidophilic cells and vacuoles in the seminiferous epithelium, and sperm production was compromised. In the epididymis, the luminal compartment was diminished, and the stroma was enlarged both in the caput and cauda; in the cauda, the epithelial compartment was enlarged; the transit time of spermatozoa through this organ was diminished. Testosterone production was lowered. Spermatozoa's motility, mitochondrial activation, and acrosomal integrity were impaired, and several alterations in morphology were observed. After the recovery period, testicular and epididymal weight was restored. Tissue remodulation was observed in the epididymis, but the spermatozoa's transit time in this organ was not altered. Sperm and testosterone production, spermatozoa motility, mitochondrial activation, and acrosomal integrity were also restored. However, testicular histological alterations and spermatic morphological abnormalities were maintained in protein-restricted animals. Protein restriction during peripuberty impairs the reproductive maturation of pubertal *Wistar* rats, impairing testicular and epididymal function, with lasting effects even after dietary correction.

Introduction

Hunger and food insecurity have persisted as global challenges for decades, with numerous studies over the past 30 years centering on this critical issue.[1,2] The 2022 report on "The State of Food Security and Nutrition in the World" reveals a concerning trend: since 2019, a growing number of individuals worldwide are grappling with hunger, with Asia and Africa bearing the brunt of undernutrition.[3] Notably, even developed nations, such as the USA, have witnessed an escalating issue of food insecurity ever since the onset of the COVID-19 pandemic,[4] particularly affecting college students.[5,6] Moderate levels of food insecurity have been linked to malnutrition,[3] which is defined as a deficiency, excess, or imbalance of nutrients in a diet, resulting in measurable effects on one's body composition and function.[7] In accordance with the Barker hypothesis, adult-onset diseases may find their origins in insults sustained during pivotal developmental windows, encompassing in utero development, childhood, and peripuberty.[8,9]

Prior investigations have delineated both immediate and delayed effects resulting from protein restriction during peripuberty. There is compelling evidence that a low-protein diet during this critical period induces metabolic dysfunction[10,11] hypertension, and sustained sympathetic arousal in adult *Wistar* rats.[12] Experimental programming models of protein restriction during the perinatal period have further demonstrated its association with redox imbalance in both the brainstem[13,14] and in the heart.[15] Concerning the male reproductive system, previous studies have established that protein restriction during pregnancy and lactation impairs offspring birth weight[16–18] and disrupts this system development by altering the

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hypothalamic-pituitary-gonadal axis.[19] Toledo *et al.*[20] have demonstrated that in utero protein restriction diminishes the number of Sertoli cells in the seminiferous tubules, thereby reducing spermatozoa production and impairing sperm quality.

A study from the late 1970s revealed that an isocaloric protein-restricted diet during peripuberty led to hypoandrogenism and abnormal regulation of the hypothalamus-pituitary-gonadal axis.[21] Another study from the same group indicated that protein-restricted (9% of proteins) Sprague-Dawley rats, postnatal day (PND) 80 onward, exhibited no discernible alterations in system maturation, given no histological alterations were observed in the testis and spermatids on stage 19 were found in protein-restricted animals, compared to the control group.[22] The authors themselves conclude that additional studies are imperative to ascertain whether metabolic programming occurs in this experimental model or not. Few studies have elucidated the delayed effects of a low-protein diet on the maturation of the male reproductive system during peripuberty.

Given the evidence presented and recognizing the significance of peripuberty as a critical developmental phase, restricting protein intake during this period could potentially impair the maturation of the male reproductive system, with possible long-term consequences in adulthood. Therefore, the objective of this study is to evaluate both the immediate and delayed effects of a 30-day low-protein diet on the postnatal development of the male reproductive system.

Materials and methods

Ethics approval

The experimental procedures described in this study were performed in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Ethics Committee on Animal Use of the State University of Maringá (CEUA/UEM protocol no. 2910011021) and by the Ethics Committee on Animal Use of the State University of Londrina (OF CIRC CEUA/UEL protocol no. 144/2019).

Animals and experimental conditions and experimental design

Forty-eight male Wistar rats from different litters at PND 25 were retrieved from the Central Animal Facility of the State University of Maringa. Subsequently, they underwent a 5-d adaptation period in the Sectorial Vivarium of the Department of Biotechnology, Genetics, and Cell Biology. Animals were housed in polypropylene cages (40 × 34 × 17 cm) three animals per cage and remained under controlled temperature (22 ± 2°C) and photoperiod (07:00–19:00, light cycle) conditions, with *ad libitum* access to water and food.

Upon reaching PND 30, the animals were stratified into four groups. Two groups were subjected to an isocaloric low-protein palletized diet (4% protein, refer to Table 1; designated as LP60 and LP120), while the remaining two received a balanced commercial control diet (20.5% protein; Nuvital®, Curitiba/PR, Brazil; refer to Table 1; denoted as NP60 and NP120).

On PND 61, rats from NP60 and LP60 groups ($n = 10$ each) underwent euthanasia via decapitation using a guillotine without anesthesia. Rats from NP120 and LP120 groups ($n = 14$ each) were transported in an air-conditioned vehicle to the Sectorial Vivarium of Physiology Science at the State University of Londrina. They were subsequentially housed three per cage under the previously described experimental conditions. For the initial 2 weeks post-transportation, minimal contact with the animals was maintained to mitigate potential stressors.

Table 1. Composition of the isocaloric low-protein and normal protein diets

Diet components	Normal protein (20.5%)	Low protein (4.0%)
Sucrose	12.72	20.00
Cornstarch	52.75	64.25
Casein (88% protein)	23.33	4.55
Mix of mineral salts	3.20	3.20
Mix of vitamins	1.60	1.60
Soybean oil	4.80	4.80
Fish oil	1.60	1.60
Total (g)	100.00	100.00

From PND 61 to PND 120, all animals received the same commercial control diet (20.5% protein; Nuvital®, Curitiba/PR, Brazil; refer to Table 1). On PND 121, rats in NP120 and LP120 groups underwent anesthesia with thiopental (THIOPENTAX®, Itapira/SP, Brazil) followed by euthanasia through heart puncture.

Testis, epididymis, and sperm collection

On PND 61, rats ($n = 10$ per group) underwent euthanasia via decapitation. The testis and epididymis were excised, with the right counterparts undergoing fixation and dedicated to histological processing ($n = 6$). Simultaneously, the left counterparts were promptly frozen and stored in a –20°C freezer for subsequent spermatic count analysis ($n = 10$). The right vas deferens were retrieved and subjected to a formaldehyde wash (10%), with the contents preserved in the refrigerator for future analysis of sperm morphology ($n = 10$). From the left vas deferens, the content was washed with phosphate-buffered saline (PBS) buffer enriched with bovine serum albumin (BSA) and destined for spermatic motility analysis ($n = 10$).

Following the recovery period, at PND 121, rats ($n = 14$ per group) were euthanized by heart puncture after anesthesia induction with 8 units of thiopental (THIOPENTAX®, Itapira/SP, Brazil). The testis and epididymis were harvested, with the right counterparts destined for histological processing ($n = 6$) or swift freezing with liquid nitrogen and storage at –80°C freezer for subsequent evaluation of oxidative damage ($n = 8$). Concurrently, the left testis and epididymis were destined for future spermatic count analysis ($n = 10$). Sperm cells from the right vas deferens were destined for analysis of sperm morphology ($n = 10$). From the left vas deferens, the content destined to spermatic motility analysis ($n = 10$). Spermatozoa extracted from the epididymal tail were utilized for the assessment of oxidative damage ($n = 8$).

Daily sperm production per testis and transit time in the epididymis

The left testis and epididymis, decapsulated ($n = 10$), underwent weighing and homogenization following the methodology outlined by Robb *et al.*,[23] with the adaptations previously described by Siervo *et al.*[24] Post-homogenization, a small aliquot of the sample was transferred to the Neubauer chamber (five fields per animal) for the enumeration of homogenization-resistant spermatids

(corresponding to stage XIX of spermatogenesis) in the testis and spermatozoa in the epididymis. To compute the daily sperm production (DSP), the concentration of homogenization-resistant spermatids per testis was divided by 6.1, which corresponds to the number of days during which mature spermatids remain in the seminiferous epithelium. Additionally, the sperm transit time through the epididymis was calculated by dividing the number of sperm in each epididymal portion by DSP.

Spermatic quality

Sperm motility assessment followed the methods of Siervo *et al.*[24] with adaptations. The left vas deferens ($n = 10$) underwent washing with 0.8 mL of PBS (137 mM NaCl, 2.68 mM KCl, 8.03 mM Na_2HPO_4 1.47 mM, pH 7.2) enriched with 0.75% BSA at 34°–37°C to obtain spermatozoa. Subsequently, a Makler counting chamber (Sefi-Medical) at the same temperature was loaded with a 10 μL aliquot of the sperm solution. Sperm motility, assessed by visual estimation (100 spermatozoa/animal) under a light microscope (Motic®, Richmond, Canada) at 100× magnification, categorized spermatozoa as motile, when active movement in this cell's tail was observed, and immotile, when no movement was seen.

Vas deferens content ($n = 10$) was removed via internal rinsing with 1.0 mL of 10% formol-saline using a needled syringe. Histological slides prepared from this solution underwent observation using an Opton photomicroscope (Berlin, Germany) at 400× magnification. One hundred spermatozoa were analyzed per animal, classifying morphology into three categories: normal morphology, head abnormalities (without characteristic curvature or isolated form, i.e., no tail attached), and tails abnormalities (broken, rolled into a spiral and isolated, i.e., no head attached, as per Fernandes *et al.*).[25]

Mitochondrial activity of the sperm ($n = 6$) was determined following the method described by Silva *et al.* (2014) with adaptations. Spermatozoa, obtained from the vas deferens by internal rinsing using a needled syringe filled with PBS enriched with 0,75% BSA, were added in microtubes containing 1 mg/mL of 3-30-diaminobenzidine (DAB) dissolved in PBS in a 1:3 (v/v) ratio and incubated at 37°C for 1 h in the dark. Smears were prepared under histological slides and fixed with 10% formaldehyde for 10 min and evaluated with a phase-contrast microscope. Cells were classified as DAB I (100% stained intermediate piece), DAB II (more than 50% staining in the intermediate part), and DAB III (less than 50% staining in the intermediate part) based on mitochondrial activity, indicating varying degrees of potential motility and fertilization capacity, from more to less capable.

Sperm acrosome status was assessed following the protocol by Silva *et al.* (2014).[26] Smears prepared onto microscope slides using fresh sperm suspension were fixed with methanol ($n = 6$). Slides were then stained with 40 μg/mL fluorescein-labeled peanut agglutinin (PNA; Sigma-Aldrich, St Louis, MO, USA) in PBS and covered with Fluoromont-G with DAPI (EMS, Hatfield, PA, USA). One hundred cells per slide were analyzed under a fluorescence Axio Zeiss microscope (Zeiss®, Oberkochen, GER). Cells had their acrosome classified as intact or damaged.

Histological analysis, testicular morphometry, and epididymal stereology

Testis and epididymis ($n = 6$) were fixed in methacarn solution (10% acetic acid, 60% methanol and 30% chloroform) before being immersed in alcohol 70%. Subsequent steps included Paraplast® wax imbibing, cutting into 5 μm sections, and staining with hematoxylin and eosin (H&E). Evaluation with a Motic microscope (Motic®, Richmond, Canada) at 100x and 400× magnification followed. For the testes, random seminiferous tubular sections (100/animal) in three nonconsecutive testis cross sections were analyzed for abnormalities, including immature germ cells in the lumen, acidophil cells, vacuoles, and tubular degeneration. In the epididymis, histological inspection was performed qualitatively in the caput-corpus and cauda of each animal as described by Favareto *et al.*[27]

For evaluation seminiferous tubule diameter and epithelium height, 40 random testicular cross sections per animal ($n = 6$) in stage IX of the seminiferous epithelium cycle underwent examination. Seminiferous tubule diameters and epithelium heights were measured using an Opton photomicroscope (Berlin, Germany) (400× magnification) and Motic software (version 3.1 for MacOS). A mean of four measures for the diameters and heights was calculated and used in the statistical analysis, aligning with the protocol by Favareto *et al.*[27] The IX stage was used given this stage is characterized by a well-defined and stable structure of the seminiferous epithelium, in which its height and the tubule's diameter are representative of the overall structural integrity.

The number of Sertoli cells with observable nuclei was determined in 20 cross sections of the seminiferous tubules per testis ($n = 6$) in each rat, employing a light microscope at 400× magnification, following the procedure by Nassr *et al.*[28] The tubules were evaluated at stages VII–VIII of spermatogenesis, given the crucial role of Sertolli cells in spermiation and acrosomal development, which occurs during these stages.

The number of Leydig cell nuclei was determined in 10 images of the interstitium per testis ($n = 6$) for each rat, utilizing an Opton photomicroscope (Berlin, Germany) at 400× magnification.

Stereological analysis of the epididymis involved capturing and analyzing 10 random epididymal cross sections per animal. Weibel's multipurpose graticulate, with 168 points superimposed on the images, permitted the counting of overlapping dots in the seminiferous cord or interstitial compartment. This enabled the establishment of respective proportions of each of these components in the epididymis for each experimental group, adhering to the method by Favareto *et al.*[27]

Staging of the spermatogenic cycle

Random seminiferous tubular sections (100/animal; $n = 6$) in three nonconsecutive testis cross sections were classified into four stages: I–VI, VII–VIII, IX–XIII, and XIV of the seminiferous epithelial cycle. This classification followed the criteria established by Leblond and Clermont,[29] using a photomicroscope at 400× magnification. This analysis allowed for the assessment of the proportion of staging of the seminiferous tubules.

Testosterone assay

Blood samples were collected in heparinized tubes and centrifuged at 2400 *g* for 20 min (4°C). The plasma was separated and stored at −20°C until the assay. Total plasmatic testosterone concentration was measured using chemiluminescence (Architect System, Wiesbaden, Germany) according to the manufacturer's protocol. All samples were included in the same assay to avoid inter-assay errors.

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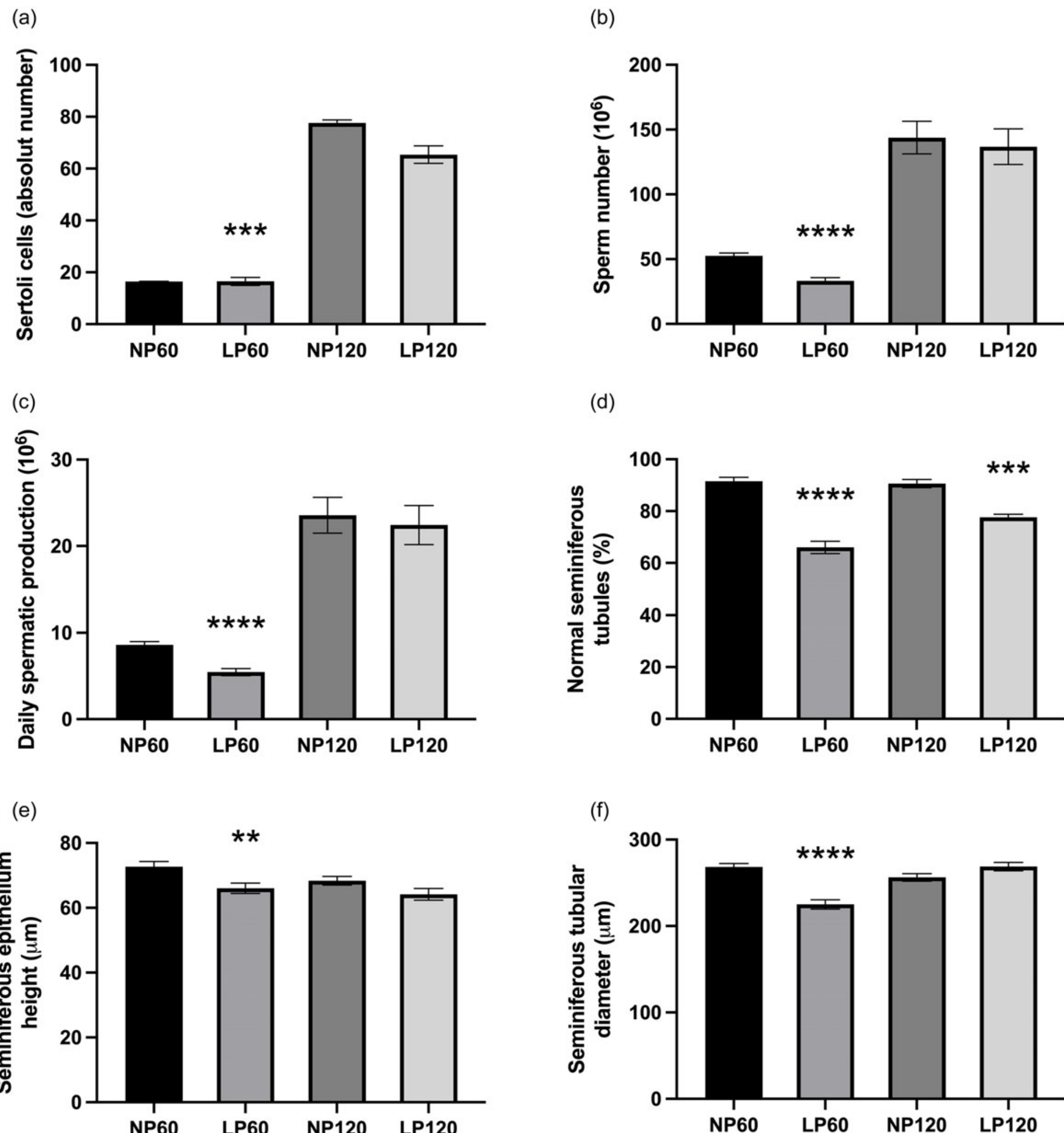

Figure 1. Sperm production, testicular morphometry and Sertoli cells count of *Wistar* rats fed a normoproteic or a low-protein diet from PND 30–60 at puberty and after a 60-d recuperation period. Data presented as mean ± SEM. Unpaired *t*-test: LP60 vs. NP60; LP120 vs. NP120. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. (***a***) Number of Sertoli cells. (***b***) Spermatic reserve in the testis. (***c***) Daily spermatic production. (***d***) Number of normal seminiferous tubules. (***e***) Seminiferous epithelium height. (***f***) Diameter of the seminiferous tubules.

Oxidative stress of the testis, epididymis, and sperm cells

Sperm collected from the epididymis tail was homogenized with and Ultraturrax homogenizer (Marconi, Piracicaba, Brazil) in 1 mL of PBS (pH 7.2) and centrifuged at 9500 *g* for 10 min at 4°C, as described by Erthal *et al.* (2022). Tissue samples from the testis and epididymis head and tail were homogenized in 500 mL of PBS (pH 7.2) and centrifuged at 9500 *g* for 10 min at 4°C. Protein quantification of tissular and sperm samples was determined by the Bradford method, using BSA as a standard.[30] Samples were then normalized to 1 mg of protein per mL of solution, following the methodology described by Erthal *et al.* (2022), and utilized for the following analyses: lipid peroxidation (TBARS), reduced glutathione (GSH), catalase (CAT) activity, glutathione S-transferase (GST) activity, and superoxide dismutase (SOD) activity.

Lipid peroxidation

Lipid peroxidation was measured through lipid hydroperoxide, a step of fatty acids oxidation.[32,33] Hydroperoxide was determined through ferric oxidation (Fe^{2+}; ammonious ferric sulfate). The Fe^{3+} ion that is formed reacts with xylenol orange, forming a colorful compound detected through its absorbance at 560 nm.[34] Results were expressed as nmol/protein mg.

Reduced glutathione concentration

The concentration of GSH was determined as described by Rahman *et al.*,[35] with adaptations made by Erthal *et al.*[31] GSH levels were measured by adding 5,5,-dithiobis-2-nitrobenzoidc acid into the normalized samples, resulting in a yellow solution, with its absorbance read at 412 nm. The results are expressed as

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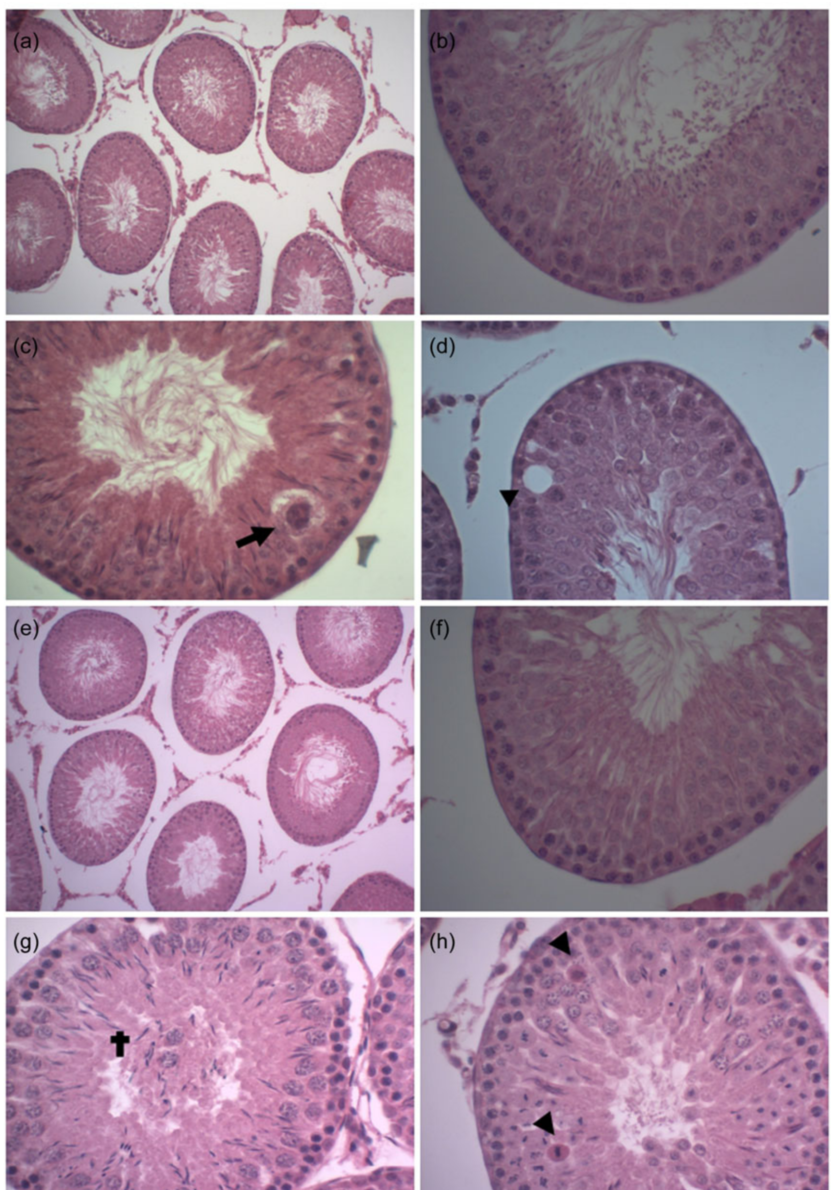

Figure 2. Histological analysis of the testis of *Wistar* rats fed a normoproteic or a low-protein diet from PND 30–60 at puberty and after a 60-d recuperation period. (***a*** and ***b***) Photomicrographs of NP60 testes, showing the preserved architecture of the seminiferous tubules. (***c***) Gigantic multinucleated cell, indicated by a thin arrow (LP60). (***d***) Presence of vacuole in the seminiferous epithelium, indicated by a bold arrow (LP60). (***e*** and ***f***) Photomicrographs of NP120 testes. Preserved architecture of the seminiferous tubules. (***g***) Round nucleated cells in lumen of the seminiferous tubule, indicated by a white arrow (LP120). (***h***.) Presence of acidophilic cells in the seminiferous epithelium, indicated by short arrows (LP120).

micromoles/mg of protein. Results were expressed as μg/ protein mg.

Catalase activity

The enzymatic activity of CAT was determined by degradation of hydrogen peroxide into water and oxygen. Using the normalized solution (1 mg/mL), 297 μL of the reaction medium was added in a UV4 microplate, and the absorbance was read at 240 nm for 60 s.[36] Results were expressed as mM/min/protein mg.

Superoxide dismutase activity

The enzymatic activity of SOD was determined as described by Senthikulmar *et al.*[37] with some adaptations made by Erthal *et al.*[31] The enzyme present in the normalized homogenates (1 mg/mL) was added to a microplate in which a reaction mixture containing sodium carbonate buffer (50 mM, pH 10.2), nitroblue tetrazolium (NBT) (96 mM), and Triton X-100 (0.6%), previously incubated for 2 min with sodium hydrochloride hydroxylamine ($NH_2OH.HCl$) (20 mM, pH 6.0), was added. The final volume in the microplate well was adjusted to 200 μL. The method is based on the quantification of complexes formed by superoxide anions in addition to NBT and $NH_2OH.HCl$ of yellowish color, with the reduction of NBT, forming a bluish solution, which is then read at 560 nm for 2 min at intervals of 15 s. Results were expressed as U/protein mg.

Glutathione S-transferase activity

GST activity was determined as described by Keen *et al.*[38] The method is based in the formation of a thioether from the interaction of GSH with 1,chloro-2,4-dinitrobenzene, with its absorbance read at 340 nm during 5 min at 40 s intervals. Values were expressed in mM/protein mg.

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Table 2. Spermatogenesis kinetics of *Wistar* rats fed a normoproteic or a low-protein diet from PND 30–60 at puberty and after a 60-d recuperation period

Spermatogenesis kinetics	Experimental groups			
	NP_{60}	LP_{60}	NP_{120}	LP_{120}
Stages I–VI	62.83 ± 1.96	43.17 ± 0.73***	56.00 ± 0.89	49.40 ± 1.50
Stages VII–VIII	17.00 ± 1.53	13.50 ± 2.57	18.00 ± 1.26	20.60 ± 1.72
Stages IX–XIII	26.33 ± 1.54	39.50 ± 2.64**	21.20 ± 0.49	28.30 ± 1.16***
Stage XIV	3.83 ± 0.31	3.83 ± 0.95	4.80 ± 0.92	1.80 ± 0.37*

PND, postnatal day; NP, normoproteic group; LP, low-protein group.
Data presented as mean ± SEM. Unpaired *t*-test.
*$p < 0.5$.
**$p < 0.01$.
***$p < 0.001$.

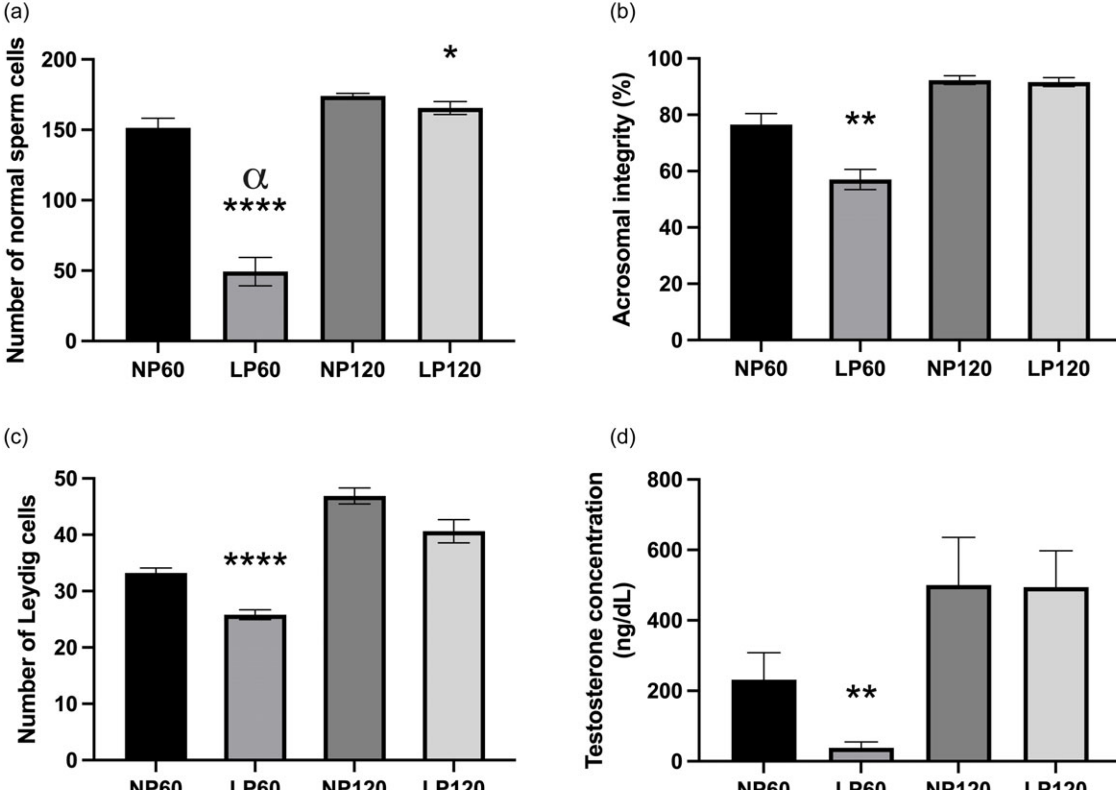

Figure 3. Spermatic parameters, Leydig cell count and testosterone concentration of *Wistar* rats fed a normoproteic or a low-protein diet from PND 30–60 at puberty and after a 60-d recuperation period. Data presented as mean ± SEM. Unpaired *t*-test or Mann–Whitney test (α): LP60 vs. NP60; LP120 vs. NP120. *$p < 0.5$; **$p < 0.01$; ****$p < 0.0001$. (***a***) Morphological analysis of spermatozoa collected from the vas deferens. (***b***) Acrosomal integrity of spermatozoa collected from the spermatic duct. (***c***) Number of Leydig cells in the interstitium. (***d***) Plasmatic testosterone concentration.

Statistical analysis

All parameters were submitted to the Shapiro–Wilk test for normality and thus classified into parametric or nonparametric data. Data were compared using an unpaired *t*-test or the nonparametric Mann–Whitney test. Differences were considered significant when $p < 0.05$.

Results

Daily sperm production and number of Sertoli cells

The absolute count of spermatids found in the testis (Fig. 1b), daily sperm production (Fig. 1c), and the number of Sertoli cells (Fig. 1a) exhibited a significant reduction in LP60 animals. However, these parameters were restored in LP120 animals. A notable 36% decrease was observed in the daily spermatic production of LP60 animals compared to NP60 animals.

Testicular morphometry, histological analysis, and staging of the spermatogenic cycle

In a histological analysis, a low-protein diet impaired normal testicular architecture during puberty and adult life, evident from the decrease in the number of normal tubules in LP60 and LP120 animals (Fig. 1d). Alterations such as the presence of vacuoles, round cells in the light of the seminiferous tubules, gigantic

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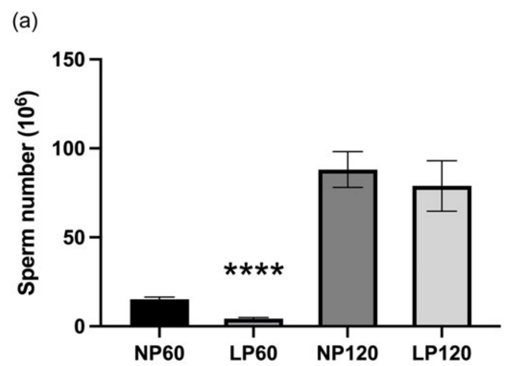
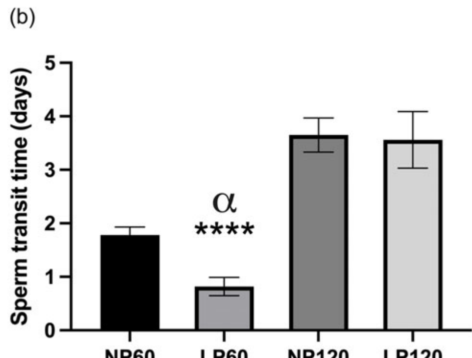
Table 3. Epididymal stereology of *Wistar* rats fed a normoproteic or a low-protein diet from PND 30–60 at puberty and after a 60-d recuperation period

	Experimental groups			
Epididymal parameters	NP_{60}	LP_{60}	NP_{120}	LP_{120}
Caput				
Epididymal compartments				
Lumen	35.42 ± 2.77	26.24 ± 1.63*(α)	20.02 ± 1.60	45.56 ± 3.19(α)
Stroma	66.04 ± 2.22	76.30 ± 1.60***	73.54 ± 2.43	65.84 ± 2.38*(α)
Epithelium	66.54 ± 2.11	65.40 ± 1.82(α)	65.36 ± 1.85	56.60 ± 2.28**
Cauda				
Epididymal compartments				
Lumen	58.38 ± 1.65	16.15 ± 1.21****(α)	85.74 ± 3.12	88.02 ± 2.85(α)
Stroma	51.92 ± 1.79	64.61 ± 2.56****	49.08 ± 2.32	48.66 ± 2.50(α)
Epithelium	58.08 ± 1.24	87.27 ± 2.39****	33.18 ± 2.01	31.28 ± 1.85(α)

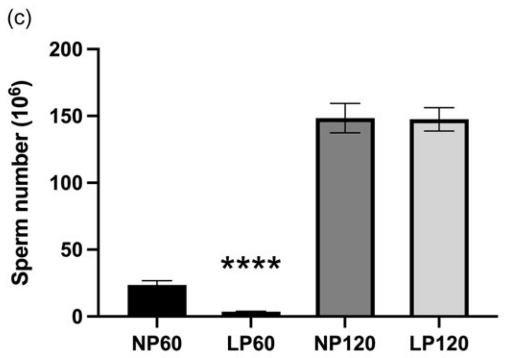
PND, postnatal day; NP, normoproteic group; LP, low-protein group.
Data presented as mean ± SEM. Unpaired *t*-test or Mann–Whitney test (*α*).
*$p < 0.5$.
**$p < 0.01$.
***$p < 0.001$.
****$p < 0.0001$.

Epididymal caput





Epididymal cauda



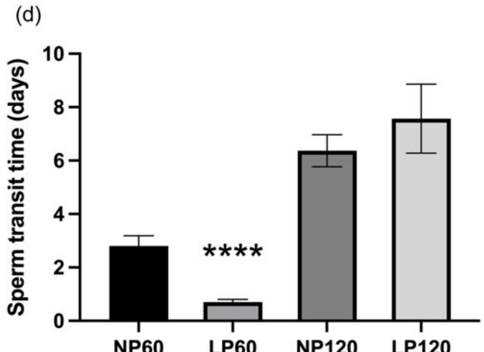


Figure 4. Sperm count in the epididymis of *Wistar* rats fed a normoproteic or a low-protein diet from PND 30–60 at puberty and after a 60-d recuperation period. Data presented as mean ± SEM. Unpaired *t*-test or Mann–Whitney test (*α*): LP60 vs. NP60; LP120 vs. NP120. ****$p < 0.0001$. (***a***) Number of spermatozoa in the epididymal caput. (***b***) Spermatozoa's transit time through the epididymal caput. (***c***) Number of spermatozoa in the epididymal cauda. (***d***) Spermatozoa's transit time through the epididymal cauda.

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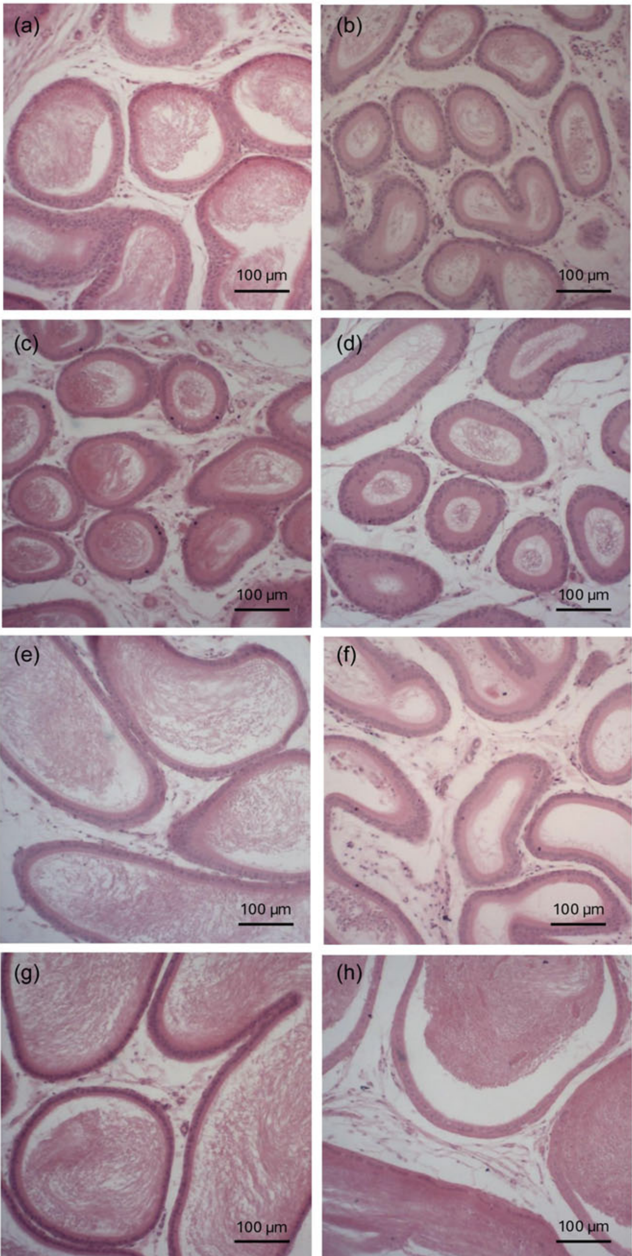

Figure 5. Histological analysis of the epididymis of *Wistar* rats fed a normoproteic or a low-protein diet from PND 30–60 at puberty and after a 60-d recuperation period. (***a***) Photomicrograph of the epididymal caput in NP60 animals, showing the preserved architecture of the epididymal duct. (***b***) Epididymal caput of a LP60 animal. (***c***) Epididymal caput of an NP120 animal. (***d***) Photomicrographs of the epididymal caput in LP120. (***e***.) Photomicrograph of the epididymal cauda in NP60 animals, showing the preserved architecture of the epididymal duct. (***f***) Epididymal cauda in LP60. (***g***) Epididymal cauda in NP120. (***h***) Epididymal cauda in LP120.

multinucleated cells, and acidophilic cells were observed in all animals with restricted diets, both during puberty and in adult life. These alterations are available for visualization in Fig. 2. The seminiferous epithelium height (Fig. 1e) and tubular diameter (Fig. 1f) were diminished in LP60 animals but restored in LP120 animals.

The spermatogenic cycle was also impaired in protein-restricted animals, as seen in Table 2. Fewer tubules in stages I–VII and more tubules in stages IX–XIII were observed in the testis of LP60 animals, while more tubules in stages IX–XIII and fewer tubules in stage XIV of the spermatogenic cycle were observed in LP120 animals.

Leydig cell count and testosterone concentration

The number of Leydig cells (Fig. 3c) within the interstitium of LP60 animals and plasmatic testosterone concentration (Fig. 3d) were also diminished by a low-protein diet but restored in adult life. The reduction in the number of Leydig cells in LP60 animals was 22% compared to NP60. For testosterone concentration, the reduction was 83%.

Epididymal transit of sperm cells, stereology, and histological analysis

The number of sperm cells found in both epididymal regions (caput and cauda; Fig. 4a, 4c, respectively) and the time of spermatic transit (Fig. 4b, 4d) were considerably diminish,ed in LP60 animals. However, after a 60-d recovery period, these parameters were restored.

Tissue remodulation was observed in the caput of LP60 and LP120 animals and in the cauda of only LP60 animals. In the caput region, a reduction in the luminal compartment and an enlargement in the stromal compartment were noted, while in the cauda, a diminution in the luminal compartment and an enlargement in the stromal and epithelial compartments were observed in LP60 animals. In the caput of LP120 animals, a reduction in the stromal compartment and an enlargement in the epithelial compartment were observed, as seen in Table 3.

Regarding the histological analysis, no alterations in the epididymis were observed in the protein-restricted animals (Fig. 5).

Spermatic quality

Sperm motility (Fig. 6a), mitochondrial activity (Fig. 6b–d), and acrosomal integrity (Fig. 3b) were adversely affected by a low-protein diet in juvenile rats but showed restoration after a 60-d recovery period. However, sperm morphology (Fig. 3a) was also impacted by protein restriction, with no recovery observed after the recovery period.

Evaluation of oxidative stress

TBARS

The concentration of TBARS in adult animals was altered only in the epididymal caput (Fig. 7a), but not in the epididymal cauda (Fig. 7b), testis or spermatozoa (Fig. 8a, 8b, respectively).

Antioxidant capacity

The antioxidant capacity in the testis, evaluated by GSH concentration (Fig. 8d) and GST (Fig. 8f), CAT (Fig. 8j), and SOD (Fig. 8h) activities, was not impaired by a protein-restricted diet. In the epididymal caput, only GST activity (Fig. 7e) was diminished, and GSH levels (Fig. 7c) and CAT (Fig. 7i) and SOD (Fig. 7g) activities were not altered in adult animals. While in the caudal region of this organ, no alterations were found among experimental groups, as disposed in Fig. 7d, 7f, 7h, 7j. In sperm cells, GSH levels (Fig. 8c) and GST (Fig. 8e) and SOD (Fig. 8g) activities were not altered by protein restriction; however, CAT activity (Fig. 8i) was found increased in LP120 animals' spermatozoa.

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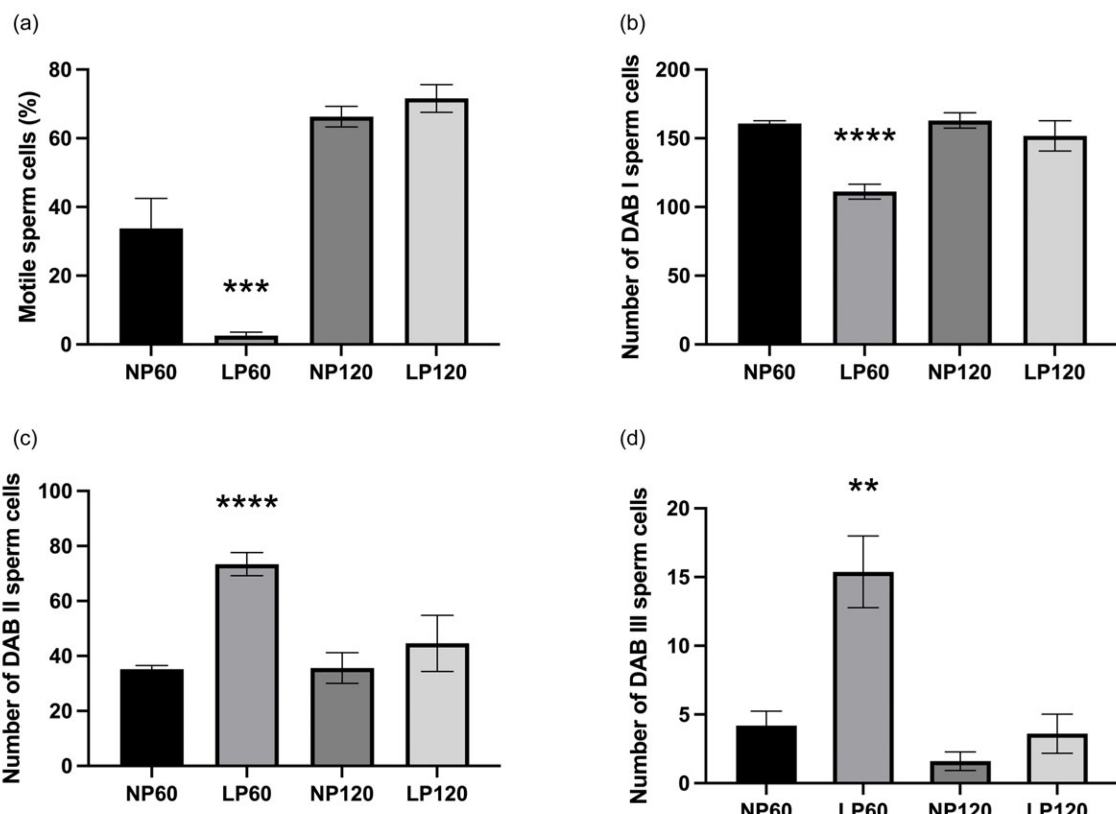

Figure 6. Spermatic parameters related to the epididymis of *Wistar* rats fed a normoproteic or a low-protein diet from PND 30–60 at puberty and after a 60-d recuperation period. Data presented as mean ± SEM. Unpaired *t*-test: LP60 vs. NP60; LP120 vs. NP120. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. (***a***) Number of motile sperm cells from the vas deferens. (***b***) Number of DAB I cells, mid-piece 100% stained by DAB. (***c***) Number of DAB II cells, 50% or more staining in the mid-piece. (***d***) Number of DAB III cells, less than 50% staining in the mid-piece.

Discussion

In this study, we demonstrated that a low-protein diet during the peripubertal period impairs the morphology and the physiology of the testis and epididymis of *Wistar* rats. This impairment was observed in the pubertal animals, at PND 60, and some of the abnormalities were carried onto adult life, since some of this damage was visible also in adult animals, at PND 120.

It has been described that a low-protein diet (9% protein) for 4 weeks during the peripubertal period decreases the testicular weight and impairs testosterone output.[21] In the present study, the decrease in the number of Leydig and Sertoli cells in LP60 may be influencing the testicular parenchymal weight, while also impairing testicular testosterone production and spermatogenesis. Leydig cells are responsible for testosterone production, the latter playing a central role in testicular maturation and spermatogenesis stimulation.[39] Sertoli cells, on the other hand, are known for their morphophysiological support in spermatogenesis, as they have cytoplasmatic structures in which the cells from the spermatogenic lineage are supported from the beginning to the end of the spermatogenic process and thus coordinated by testosterone levels.[40] Previous studies showed that germ cells[41] and Sertoli cells are required for the retention of the Leydig cell population.[42] Thus, we observed that sperm production was impaired in LP60 due to the morphophysiological abnormalities observed in the testis.

Leydig cells' testosterone output depends on many factors, but cholesterol availability and LH stimulation are vital in this process.[43] By negative feedback mechanism, testosterone levels control luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion by the pituitary, which, in turn, control gonadotropin-releasing hormone (GnRH) output by the hypothalamus.[44] Polkowska *et al.*[45] demonstrated that a protein-restricted isocaloric diet (8%) after 24 weeks of consumption impaired LH levels in female lambs. Other studies from the 1980s also demonstrated that protein restriction for 4 weeks during peripuberty impairs FSH and LH production, leading to hypoandrogenism in Sprague-Dawley rats.[21,46] Data already published using the same experimental design demonstrated that cholesterol levels were not decreased by a protein-restricted diet,[11] thus corroborating with the findings of Glass *et al.*[21] and Polkowska *et al.*,[45] indicating that an impairment in the hypothalamus-pituitary-gonadal axis is occurring in LP60 animals.

Spermatogenesis is a complex process coordinated by Sertoli cells and testosterone levels.[47,48] Alterations in number and function of these cells can compromise the spermatogenesis process.[49] Also, alterations in the stages of the spermatogenic cycle, such as the stagnation during the stages IX–XIII, which present one generation of spermatids, and an acceleration in stages I–VI, showing two

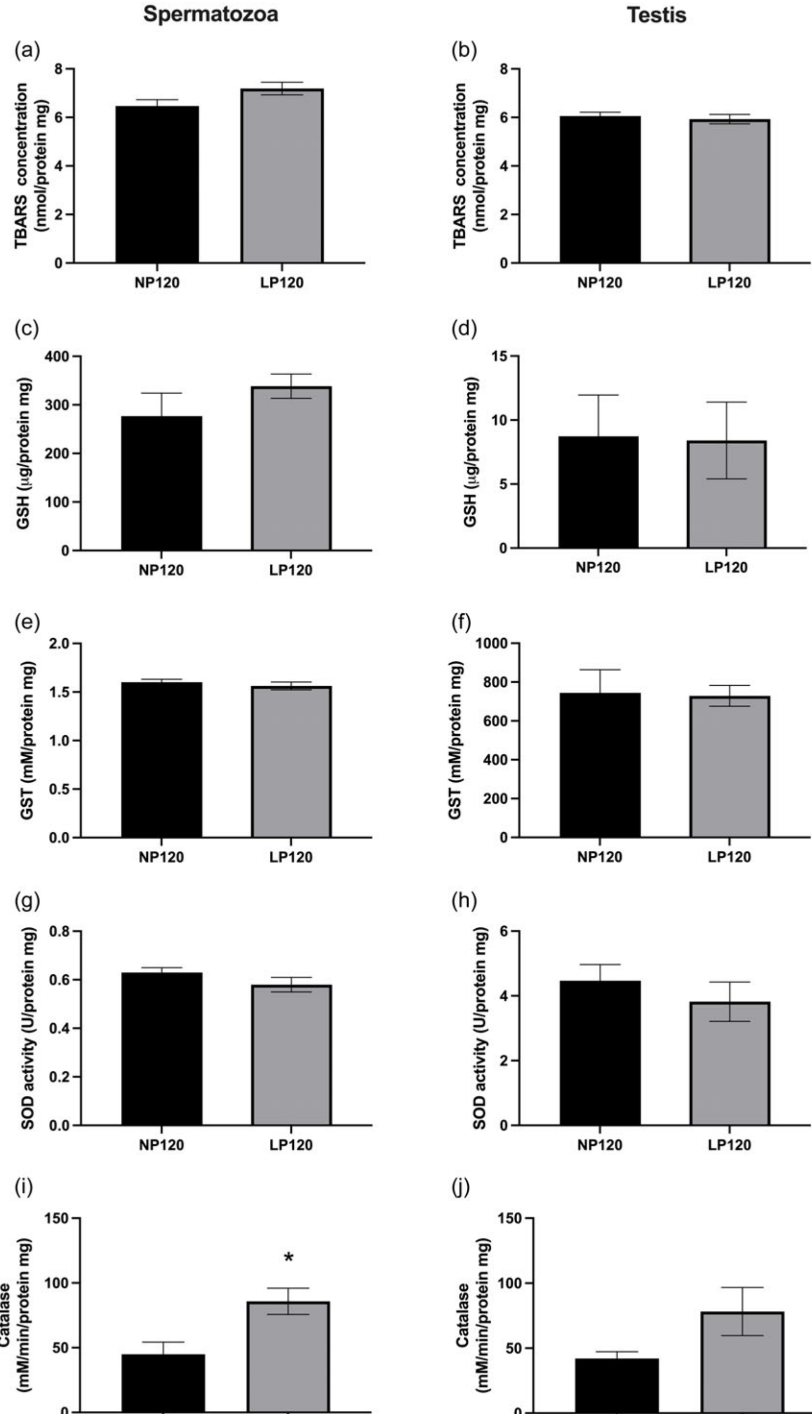

Figure 7. Oxidative profile in the epididymis of *Wistar* rats fed a normoproteic or a low-protein diet from PND 30–60 after a 60-d recuperation period. Data presented as mean ± SEM. Unpaired *t*-test. (a and b) Quantification of substances reactive to tiobarbituric acid in the epididymal caput (***a***) and in the epididymal cauda (***b***). (c and d) Quantification of reduced glutathione (GSH) in the epididymal caput (***c***) and in the epididymal cauda (***d***). (e and f) Glutathione S-transferase (GST) activity in the epididymal caput (***e***) and in the epididymal cauda (***f***). (g and h) Superoxide dismutase (SOD) activity in the epididymal caput (***g***) and in the epididymal cauda (***h***). (i and j) Catalase activity in the epididymal caput (***i***) and in the epididymal cauda (***j***).

generations of spermatids, impact directly in the sperm count and daily sperm production, as observed in LP60 animals.

Apart from its main role in spermatogenesis, Sertoli cells also play a key role in spermiogenesis. The latter is a complex process that comprehends the spermatids' elongation, the condensation of its nuclei, the development of its tail and of the acrosome, and the loss of the cytoplasmatic excess, culminating in the acquirement of the sperm cell characteristic morphology.[50] In light of these facts, it becomes evident that the diminution in the number of Sertoli cells impaired the sperm cells morphology and the acrosomal integrity of LP60. In the normal architecture of the seminiferous tubules, Sertoli cells are columnar shaped, its base lies on the basal lamina of the seminiferous tubule, and its apex is in touch with the light of this structure.[51] The diminution in the seminiferous epithelium height and tubular diameter, in the current study, is directly correlated with the diminution in number of Sertoli cells and with the vacuolization and degeneration observed in that structure in LP60, given that these cells are required for the development and maintenance of the seminiferous epithelium.[39]

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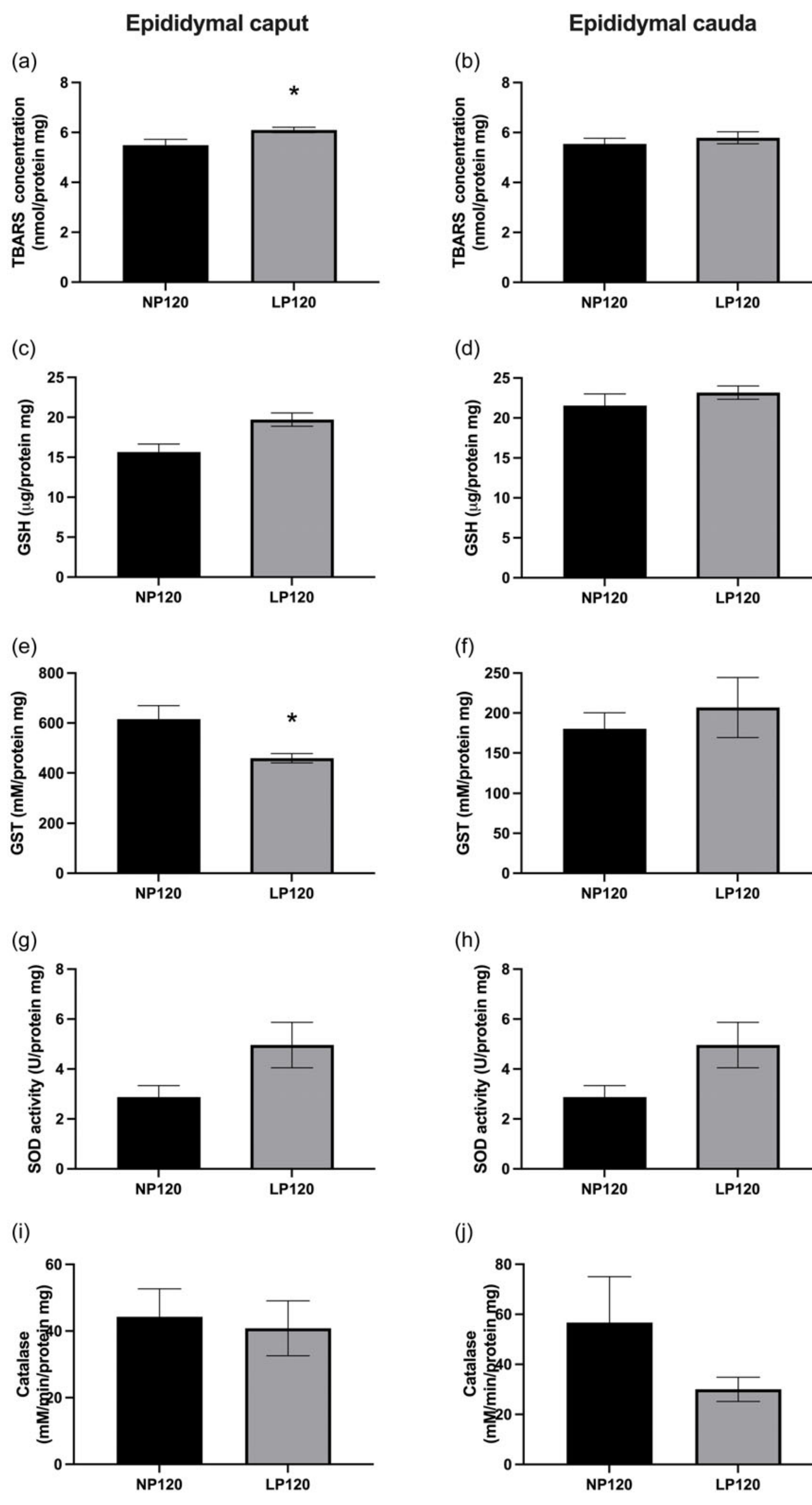

Figure 8. Oxidative profile in the sperm cells and testis of *Wistar* rats fed a normoproteic or a low-protein diet from PND 30–60 after a 60-d recuperation period. Data presented as mean ± SEM. Unpaired *t*-test. (a and b) Quantification of substances reactive to tiobarbituric acid in spermatozoa (***a***) and in the testis (***b***). (c and d) Quantification of reduced glutathione (GSH) in spermatozoa (***c***) and in the testis (***d***). (e and f) Glutathione S-transferase (GST) activity in spermatozoa (***e***) and in the testis (***f***). (g and h) Superoxide dismutase (SOD) activity in spermatozoa (***g***) and in the testis (***h***). (i and j) Catalase activity in spermatozoa (***i***) and in the testis (***j***).

Regarding the abnormalities sustained in animals that underwent a 60-d recovery period with normocaloric diet, such as histological findings in the testes, a stagnation in spermatogenesis, and impaired spermatozoa's morphology, it is noted that some of the alterations presented by the animals right after the challenge are carried into adult life. Chen *et al.*[52] have linked histological alterations in the testis to an impairment in the spermatogenic process, culminating in the production of morphologically impaired spermatozoa. Corroborating with these findings, even though after a recovery period the Sertoli cell count was normalized, the spermatogenic process was stagnated at stages IX–XIII and histological abnormalities were still present in the

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testis, compromising the production of morphologically normal sperm cells in LP120 animals, without impairing the daily sperm production.

Sertoli and Leydig cell numbers were immediately diminished by a low-protein diet; however, these cell populations were replenished after the 60-d recovery period. Traditionally, it was thought that Sertoli cells ceased proliferation before puberty.[53–55] However, recent data in the literature demonstrate that Sertoli cells are capable of proliferation during adulthood under certain conditions,[56,57] allowing for replenishment during the recovery period. Leydig cells' growth and differentiation is usually complete around PND 70, and in adulthood, their numbers are sustained by stem Leydig cells within the interstitium.[58]. These stem cells are also responsible repopulating the interstitium under some circumstances.[59,60] Therefore, the replenishment of Leydig cells in adult animals in comparison to pubertal ones relies on the normal proliferation of this cell population and the proliferation of Leydig stem cells.

Regarding the epididymal findings, the transit time of sperm cells in the epididymis is a result of the contractile activity of smooth muscle cells contained in this organ, modulated by androgens and autonomous neurotransmitters.[61] Thus, the augment in the sperm cells' transit time in this organ is also related to the diminished testosterone levels in LP60. The epididymal epithelium is responsible for the secretion of many proteins and factors that allow sperm cells maturation, in this sense, alterations in the pattern of secretion may impair sperm quality.[62,63] The tissue remodulation observed in the epididymal cauda, where spermatozoa are stored,[63] indicates that the epithelial cells in this portion of the epididymal duct may be overloaded. Given its enlargement in LP60, thus might not be able to provide the proper environment for spermatozoa. Also, the replacement of the luminal compartment with stromal tissue in LP60, both in the caput and cauda, reflects the diminished sperm production and diminution in transit time, given there are lesser cells migrating through the epididymal lumen in these animals. Interestingly, tissue remodulation is also observed in LP120 animals, evidencing that the epididymal organogenic process, which occurs during the peripubertal period, is compromised even after the protein consumption normalization.

The sperm cells' transit time in this organ is very important for the development of some spermatic parameters, such as the acquirement of motility.[64,65] For proper spermatozoa motility, it is vital that mitochondrial activation occurs in its mid-piece, offering sperm cells adequate quantities of ATP.[66] In this study, we observed a loss of sperm motility in association with impaired mitochondrial activation in these cells in LP60, as evidenced by a decrease in the number of fully stained mitochondrial sheath (DAB I) cells. Thus, the diminution of mitochondrial activity and in motility of sperm cells is closely related to the slowness of these cells' transit through the epididymis.

The male reproductive system is known to mature by the end of puberty, which, according to Ojeda *et al.*,[67] occurs between PND 55 and 65. The key to understanding why most of the abnormalities observed in LP60 animals were not sustained after a 60-d recovery period might reside in the impairment of the hypothalamus-pituitary-gonadal axis in LP60 animals and in the length of a full spermatogenic cycle, of approximately 60 d in *Wistar* rats.[68] We suggest that, given the impairment of hypothalamus-pituitary-gonadal axis during peripuberty, the testis and epididymis did not achieve its full development when it was expected to, as demonstrated by the abnormalities observed in LP60 animals. Once the challenge is withdrawn and protein availability is at normal levels, FSH and LH concentrations are risen to normal, testosterone production is normalized, and then the maturation of the reproductive system begins. An indicator of this is the rise of testosterone to normal levels observed in LP120 animals and the normalization of most sperm parameters after a recovery period, which lasted as much as a full spermatogenic cycle. Thus, only morphological abnormalities were found in LP120 sperm cells since all the severely damaged cells were reabsorbed in the epididymis or vas deferens[69] and new cells were produced under the conditions previously described.

In conclusion, protein restriction during the peripubertal period causes immediate damage to the testis, epididymis, and sperm cells, being that some of abnormalities are sustained into adult life even after protein uptake is normalized. This highlights the importance of a balanced nutrition during key developmental windows, such as peripuberty.

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Competing interests. None.

Ethical standard. The experimental procedures described in this study were performed in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Ethics Committee on Animal Use of the State University of Maringá (CEUA/UEM protocol no. 2910011021) and by the Ethics Committee on Animal Use of the State University of Londrina (OF CIRC CEUA/UEL protocol no. 144/2019).

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