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Protein restriction during intrauterine and lactation periods: effects on testicular development in pre-puberty rats

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ABSTRACT. Current study investigates the effects of maternal protein restriction during pregnancy and lactation on the testis of immature rats. Female Wistar rats were mated and, after confirming pregnancy, they were divided into two groups: undernourished group (UG) fed on a diet with casein 8%, and control group (CG) fed on a diet with casein 17 %, during pregnancy and lactation. After weaning, male offspring from the two experimental groups were fed on normal diet up to 35 days old when they were euthanized. Body and testicular weights decreased in UG when compared to those in CG. Volumetric density of seminiferous tubules was higher in CG whilst intertubular space increased in UG. The number of lumenated seminiferous tubules was higher in CG than in UG. Tubular diameter, seminiferous epithelium height and total length of seminiferous tubules were lower in UG. Round spermatids were frequently found in seminiferous tubules cross-section of CG. On the other hand, spermatocytes I in prophases was the germ cell commonly found in seminiferous tubules cross-sections of UG. Undernutrition during pregnancy and lactation period of the male Wistar rats altered the morphometric testicular parameters related to tubular compartment and delayed the onset of spermatogenesis.

Keywords: testes, seminiferous tubules, morphometry, undernutrition

Restrição proteica durante os períodos intrauterino e lactacional: efeitos no desenvolvimento testicular de ratos pré-púberes

RESUMO. O objetivo deste estudo foi investigar o efeito da restrição proteica materna durante prenhez e lactação no testículo de ratos imaturos. Fêmeas de ratos Wistar foram acasaladas e, uma vez confirmada a gestação, foram divididas em dois grupos e alimentadas durante a prenhez e a lactação: grupo desnutrido (GD), dieta com caseína 8%, e grupo controle (GC), dieta com caseína 17%. Após o desmame, os machos de ambas as proles receberam a dieta normal até os 35 dias de idade, quando ocorreu a eutanásia. O peso corporal e o peso testicular diminuíram no GD com relação ao GC. A densidade volumétrica dos túbulos seminíferos foi maior no GC, assim como o espaço intertubular aumentou no GD. O número de túbulos seminíferos luminados foi maior no GC comparado ao GD. Diâmetro tubular, altura do epitélio seminífero e comprimento total dos túbulos seminíferos diminuíram no GD. Espermátides arredondadas foram frequentemente encontradas nas seções transversais do GC. Por outro lado, espermatócitos I em prófase foram o tipo celular mais frequentemente encontrado no GD. A desnutrição durante a gestação e a lactação em ratos Wistar machos alteraram parâmetros morfométricos testiculares relacionados ao compartimento tubular e atrasou o início da espermatogênese.

Palavras-chave: testículos, túbulos seminíferos, morfometria, desnutrição

Introduction

Undernutrition is a pathological state characterized by an imbalance between the supply of nutrient and energy and the physical demand to ensure growth, maintenance and specific functions (AHUJA; MITCH, 2004). It results in physiopathological changes first translated as functional impairment and later as biochemical and physical damage (GURMINI et al., 2005).

The diet composition of pregnant females must be provided with several specific items due to their nutrient requirements ensuring the suitable operation of physiological processes during this period. Proteins have an essential role in pregnancy maintenance and success due to its variety of

functions. Restriction in protein supply decreases fetal growth and interrupts the absorption phenomena associated with development rather than the preservation of physiology and basic protein metabolism (TOLEDO et al., 2011).

Owing to great tissue plasticity, during the pre- and post-natal period, the genome regulation may be largely modified by the nutritional environment and may produce a range of different physiological or morphological states (GUILLOTEAU et al., 2009; HOCHBERG et al., 2011). When fetal environment is poor, there is a programming that optimizes the growth of key body organs to the detriment of others. This programming may be defined as the embryonic and fetal adaptive response to a suboptimal intrauterine environment that results in permanent adverse consequences (McMILLEN; ROBINSON, 2005). Changes in reproduction capacity are examples of such programming.

Neonatal and pre-pubertal periods are critic for spermatogenesis and for male reproductive activity due the differentiation, proliferation and maturation of testicular cells. Therefore, food restriction during pregnancy and lactation might impair testicular development (RODRÍGUEZ-GONZÁLEZ et al., 2012).

Studies on food restriction during early life show modifications in testicular structure related to tubular diameter and germ cells associations, and hormonal changes, such as decreases in testosterone and gonadotropin production (ZAMBRANO et al., 2005; TOLEDO et al., 2011; RODRÍGUEZ-GONZÁLEZ et al., 2012). However, some aspects of testicular development related to protein restriction in these periods of life are still poorly explored. Current study investigates the effects of maternal protein restriction in pregnancy and lactation on testicular morphometry and spermatogenesis progress in sexually immature offspring.

Material and methods

Experimental groups

Four females Wistar rats, 120 days old and average weight 280 g, were retrieved from the Nutrition Department Vivarium of the Federal University of Pernambuco, Recife, Pernambuco State, Brazil. All female rats were mated and, after confirming pregnancy with vaginal smear, they were divided into two groups: Undernourished Group (UG), fed on a diet with low protein levels (casein 8%) and Control Group (CG), fed on a diet with normal protein levels (casein 17%), according to recommendations by the American Institute of Nutrition – AIN. One day after birth, eight male and female neonates were randomly chosen and left with their mother until 21 days old, when weaning occurred. After weaning, all animals were fed with a diet containing 17 % of casein until they were 35 days old. According to Lee et al. (1975), the rats were still sexually immature since the interval between birth and sexual maturity in the rat was approximately 50 days. During the experimental procedures, the animals were kept in cages, under 12h/12h reverse cycle (light from 18h to 6h), at 22 ± 2°C, in the vivarium of Anatomy Department Annex, CCB–UFPE. Protocol (23076.051008/2011-17) was approved by UFPE Ethics Committee for Animal Experiments.

After thirty-five days of birth, all animals were weighed and anesthetized with ketamine (50 mg kg^{-1}) and xylazine (5 mg kg^{-1}) and intracardiac paraformaldehyde solution 4% in a phosphate buffer (pH 7.2) was perfused. Testes were collected, weighed, sectioned and post-fixed by immersion in the same fixative. The fragments were routinely processed in paraffin embedding and semi-serial tissue fragments with 5μm thickness were stained with hematoxylin-eosin. According to França and Russel (1998), linear shrinkage in tissue inclusion is around 15% when testes are embedded in paraffin and from 3 to 5% when embedded in plastic resin. A 15% mean correction was added to each animal to tubular diameter, epithelial height and lumen diameter measurements. The gonadosomatic index (GSI = [testicular weight / body weight] x 100) was calculated from body and testicular weights obtained on the day they were euthanized. GSI is the ratio in percentage between testicular weight and body weight (CALDEIRA et al., 2010).

Tubular diameter and total length of seminiferous tubules

The diameter of 30 randomly selected round seminiferous tubules per animal was obtained and analyzed by Leica image analysis system (Leica DM500) at 100X magnification. The tubular diameter was performed by two diametrically opposite measurements. The total length of seminiferous tubules (TLST) per testis, expressed in meters, was obtained by dividing seminiferous tubule absolute volume (STAV) by r^2 (r = diameter / 2) and π value: $TLST = STAV/\pi R^2$.

Volumetric density of testicular tissues

The volume density of testicular component was obtained by point counting with systematic allocation through micrometer reticule (Olympus U-OCMSQ 10mm/100) with 441 intersection

points on histological preparation of the testis in 400X magnification. Fifteen fields were randomly counted, totalizing 6615 points for each animal. The seminiferous tubules volume expressed in μL was established from the product of volume density (%) and testicular liquid weight calculated in milligrams (mg). The value of testicular liquid weight was obtained by subtracting 6.5 % (relative to the albuginea) of the testicular gross weight. Testis weight was considered equal to its volume because testicular density is approximately 1.03 to 1.04.

Qualitative analysis of testicular parenchyma

The testicular parenchyma were analyzed, taking into consideration the different stages of seminiferous tubules lumen appearance. Ten regions of testicular parenchyma per animal were randomly selected and analyzed under 200X magnification. Seminiferous tubules in an early stage of lumen appearance, partly and totally luminated tubules, were counted in each region. Thirty tubules were selected per animal in both groups to determine the most developed germ cell type present in seminiferous epithelium. Germ cells were analyzed using bright field microscopy for morphological characterization of cell types, following Sachetim (1999).

Statistical analysis

All data were analyzed by Mann-Whitney test with Sigmastat for Windows 2.0. Data were given by mean and standard deviation, at $p < 0.05$ significance.

Results

Body and testis weight and GSI

The reduction of protein levels in current experiment caused a reduction of the body weight close to 36%, while testicular weight was reduced approximately by 39%. However, there was no difference in the gonadosomatic index between the groups (Figure 1).

Testicular morphometry

Results showed that the reduction of casein levels to 8% in the undernourished group caused a decrease in tubular diameter (UG = 122.9 ± 16.2 μm; CG = 149.4 ± 9.3 μm; p = 0.003), height of seminiferous epithelium (UG = 32.2 ± 6.9 μm; CG = 45.3 ± 1.6 μm; p = 0.002) and total length of seminiferous tubules per testis (UG = 16.5 ± 6.2 m; CG = 29.1 ± 6.0 m; p = 0.003) when compared to rates of control group.

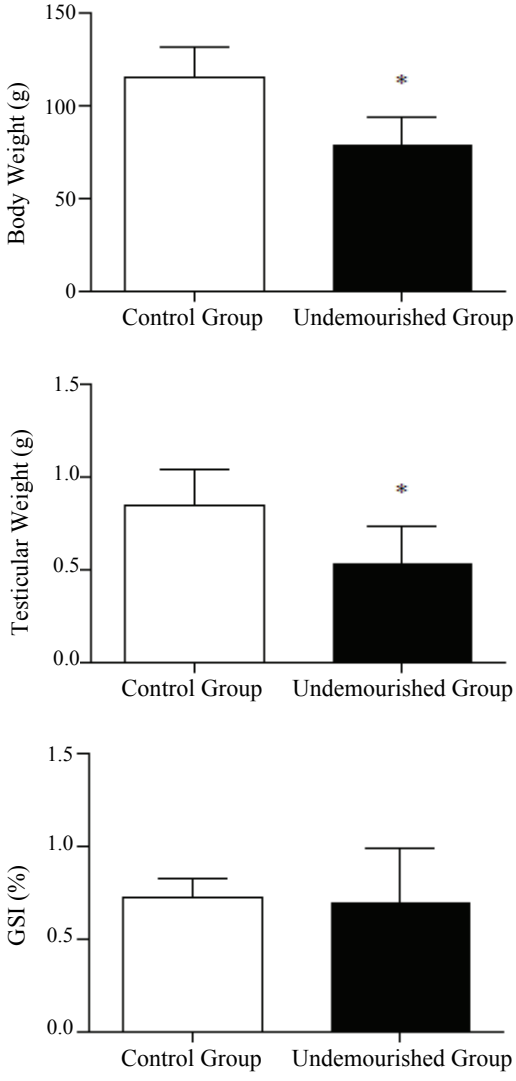

Figure 1. Effects of protein restriction during pregnancy and lactation on body weight, testis weight and gonadosomatic index (GSI) of 35-day-old Wistar rats. *Statistically significant difference ($p < 0.05$).

Nevertheless, the tubular lumen diameter showed no significant difference in experimental groups (UG = 58.7 ± 13.1 μm; CG = 57.2 ± 7.1 μm; p = 0.953). The volumetric densities (%) of the tubular and interstitial compartment in the undernourished group were, respectively, 80.6 ± 5.15 % and 19.4 ± 5.15, whereas in the control group they were 86.1 ± 1.4 % and 13.9 ± 1.4 % (p = 0.028), with a reduction in tubular area in UC (Table 1).

Relative frequency (%) of seminiferous tubules lumenated and germ cells content

The undernourished group revealed a higher frequency of tubules in the early stages of lumination (35.7 ± 37.3 %) when compared with control group (0.84 ± 0.90 %), p = 0.004.

Table 1. Histomorphometrical analysis and relative frequency (%) of testicular compartments of 35-day old Wistar rats submitted to protein restriction during pregnancy and lactation.

	Seminiferous Tubules				Volumetric Density (%)	
	Tubular diameter	Epithelium height	Lumen diameter	Tubular Length	Tubular	Interstitial
CG	149.4 ± 9.3 μm	45.3 ± 1.6 μm	57.2 ± 7.1 μm	29.1 ± 6.0 m	86.1 %	13.9 %
UG	122.9 ± 16.2 μm	32.2 ± 6.9 μm	58.7 ± 13.1 μm	16.5 ± 6.2 m	80.6 %	19.4 %
p	0.003 *	0.002 *	0.953	0.003 *	0.028 *	

*Statistically significant difference ($p < 0.05$).

After the seminiferous tubules frequency in intermediate stage was evaluated, no difference was reported between UG (7.42 ± 5.8 %) and CG (2.34 ± 1.8 %), p = 0.058.

On the other hand, totally luminated tubules were more frequent in control group (96.8± 2.4 %) than in the undernourished animals (56.9 ± 40%, p = 0.006). Further, CG had higher frequency of round spermatids (66.1 ± 10.3 %) in completely luminated tubules than in UG (44.4 ± 11.7 %), p = 0.007. However, frequency of primary spermatocytes in luminated tubules of UG was higher (55.6± 11.7 %) than CG (32.8 ± 11. 4%), p = 0.007 (Table 2).

Discussion

Decrease in body and testes weight observed in current analysis indicates that individual development is limited by the environment in which the organism grows, despite the genome (MARTIN-GRONERT; OZANNE, 2006). Feed restriction during pregnancy and/or lactation is related to reduction in body and testicular weight (ENGELBREGT et al., 2000; GENOVESE et al., 2010; TOLEDO et al., 2011).

Gonadosomatic index, or rather, the ratio between the testis size and body size, which represents the percentage of body mass allocated to the testis (CALDEIRA et al., 2010), predicts the rates of sperm production as well as the sperm function in some species (GOMENDIO et al., 2006). GSI similarity between groups could be explained because both body and testis weights had a proportional reduction in rats under protein restriction during the gestation and lactation period.

Testicular puberty is characterized by Sertoli blood-barrier, tubular lumen appearance and germ cells proliferation (SHARPE et al., 2003). In current study, due to the large amount of tubules without lumen or partially luminated in UG, it has been clearly demonstrated that protein restriction delayed lumen formation and consequently testis maturation.

Protein reduction supply during the intrauterine and postnatal periods has been related to the lateness in the establishment of puberty. Engelbregt et al. (2000) noted a delay in preputial detachment and Zambrano et al. (2005) observed a postponement of testicular descent. According to Rodríguez-González et al. (2012), animals subjected to protein restriction in similar periods to this experiment had a reduction in the number of germ cells and a delay in spermatozoa differentiation.

The tubular diameter reduction has been related to a diet with 8% casein during lactation and corroborates research by Guaragna et al. (1986). According to Hötzel et al. (1998), decrease in tubular diameter, seminiferous epithelium height and tubular length are directly related to testicular weight loss caused by nutrition. However, in current experiment, diet with low-protein levels was also used during pregnancy and may have further influenced the parameter's reduction.

The tubular volumetric density (%) and tubular diameter reduction in UG may be related to the number of Sertoli cells. The association of Sertoli cells with germ cells forms the seminiferous epithelium and the capacity to support germ cells is a species-specific pathway (HESS; FRANÇA, 2008).

Thereby, when the number of Sertoli cells has been established, the perinatal and pre-pubertal period controls the testis size, daily sperm production and the sperm count during adulthood (HESS; FRANÇA, 2008; AUHAREK et al., 2011; SCHULZ et al., 2012).

Therefore, decrease in tubular volume and diameter in 35-day-old undernourished animals might be related to numerical changes in the population of Sertoli cells.

Table 2. Relative frequency (%) of seminiferous tubules lumenation and the most developed germ cell type present in seminiferous epithelium of 35-day-old Wistar rats submitted to protein restriction during pregnancy and lactation.

	Seminiferous Tubules Lumenation (%)			Germ Cell Type (%)	
	Early stage	Intermediate stage	Totally laminated	Primary spermatocytes	Round spermatid
CG	0.84%	7.42%	96.8%	32.8%	66.1%
UG	35.7%	2.34%	56.9%	44.4%	55.6%
p	0.004 *	0.058	0.006*	0.007*	

*Statistically significant difference ($p < 0.05$).

Genovese et al. (2010) reported a decrease in the number of Sertoli cells associated to reductions in total length of seminiferous tubules in undernourished rats during pregnancy and lactation. In their assays with protein restriction in dams during pregnancy, Toledo et al. (2011) also observed reductions in the number of Sertoli cells.

Daily sperm production (DSP) is related to other parameters such as tubular diameter, volumetric density and length of seminiferous tubules (HÖTZEL et al., 1998; HESS; FRANÇA, 2008). The volumetric density (%) of seminiferous tubules, tubular diameter and epithelium height changes observed in the undernourished group may be related to DSP depletion as soon as these animals reach adulthood.

Protein restriction during the pregnancy and lactation periods delayed the emergence of round spermatids but enhanced the frequency of primary spermatocytes. Animals of both experimental groups received their corresponding diets until 21 days old, but they were euthanized at 35 days. It is known that spermatogonia from post-natal rats´ testes usually enter the seminiferous epithelium cycle after 6 days of postnatal life (CLERMONT; PEREY, 1957) to produce the first spermatogenic wave. Each seminiferous epithelium cycle in rats lasts about 13 days (CLERMONT; HARVEY, 1965).

In their experiment with protein restrictions in lambs, Carrijo Júnior et al. (2008) reported a spermatogenesis disruption characterized by a greater number of spermatogonia per tubules in undernourished animals and low frequency of spermatocytes, spermatids and spermatozoa. Lac and Lac-Scanzi (1984) showed a spermatogenesis disruption related to protein deprivation in which the testes of 63-day-old undernourished animals had the same germ cell types of pre-pubescent testis. Toledo et al. (2011) found that intrauterine protein restriction caused the reduction in the number of spermatids, sperm production and sperm number stored in the epididymis tail, which were related to a decrease in serum testosterone levels.

Gonadotropins and testosterone participated in the regulation of gonadal growth and maturation during puberty, and stimulated the initiation of spermatogenesis (MAIN et al., 2006; VERHOEVEN et al., 2010). Maternal dietary restrictions affected offspring hypothalamic-pituitary-gonadal axis, reducing LH and testosterone concentrations (ZAMBRANO et al., 2005: TOLEDO et al., 2011; RODRÍGUEZ-GONZÁLEZ et al., 2012). Combined with hormonal changes, some studies evidence delayed testicular descent, sexual development, reductions in daily sperm production and reproductive capacity (ZAMBRANO et al., 2005; TOLEDO et al., 2011; RODRÍGUEZ-GONZÁLEZ et al., 2012). Thus, changes in hormone concentrations may have influenced directly or indirectly the testicular damages observed in current study.

The protein restriction during pregnancy and lactation may cause irreversible damages on the testis, which may be related to decrease in the number of Sertoli cells. Further, restrictions in protein intake might have influenced the Sertoli cells' ability of nutritional support, resulting in a smaller number of germ cells capable of maturing.

Conclusion

The female nutritional status during pregnancy and lactation plays an important role in the pre- and post-natal testicular development. Undernutrition during these periods may change parameters related to the onset and maintenance of sperm production. Thus, spermatogenesis and sperm production will be jeopardized in adult animals when submitted to low protein levels during perinatal period.

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