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Stereological Evaluation of the Seminiferous Tubules of Rats after Maternal Undernutrition during the Lactation Period

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Key Words
Undernutrition · Lactation · Testes · Seminiferous tubules · Stereology

Abstract
The goal of this study is to evaluate, through stereological methods, some structural aspects of offspring testes whose dams were submitted to protein and energy-restricted diets during the lactation period. At birth, dams were separated into 3 groups: control group (C), receiving a diet with 23% protein; protein-restricted group (PR), receiving a diet with 8% protein; energy-restricted group (ER), receiving a diet with 23% protein in restricted quantities. At weaning, the offspring was anesthetized and perfused with formalin solution. Then, the testes were excised and processed using routine histological methods. Compared to the C group, both PR and ER groups had a significant reduction in the testis weight (PR = 65%, ER = 60%, $p < 0.01$), in the total area (PR = 23%, ER = 32%, $p < 0.01$), in the luminal area (PR = 30%, ER = 36%, $p < 0.01$), in the epithelial area (PR = 21%, ER = 27%, $p < 0.01$), and in the epithelial height (PR = 17%, ER = 23%, $p < 0.01$) of the seminiferous tubule. We conclude that maternal malnutrition during lactation leads to structural changes in the testis that could be responsible for future alterations in this organ physiology.

Introduction

Malnutrition is the most prevalent form of nutritional disorder among children in developing countries. Onís et al. [1] reported that, based on World Health Organization data, about 43% of children in developing countries suffer from malnutrition during some period in their lives. Protein malnutrition often occurs during gestation, lactation, and the first 2 years of life [2].

Some authors showed that the nutritional status of the mother, during the gestational and lactation periods, is essential to the normal growth and development in humans [3–5] or in experimental animals [6–10].

Several experimental models were designed to evaluate the effects of malnutrition in the offspring rats. Most authors studied malnutrition over the gestational period or both the gestational and lactation periods. Changes in neural development [11], insulin secretion [12] and thyroid function [13] were reported in models of malnutrition during lactation in the malnourished weaning rat.

Some authors showed that pre-pubertal malnutrition changes the serum and pituitary hormonal levels [14] and the morphological characteristics of gonadotrophs and mammotrophs [15].

Despite the fact that undernutrition-related reproductive suppression in rats is a well-documented phenomenon [15, 16], as yet, we do not have knowledge on how

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undernutrition affects the reproductive system in the offspring whose dams are submitted to malnutrition during lactation. These data would be very important to understand the potential effects of undernutrition on reproductive function.

The goal of this study is to evaluate the testicular weight, and through stereological methods, the epithelial and luminal areas, as well as the epithelial height of seminiferous tubules from testes of offspring whose dams were submitted to protein and energy-restricted diets during the lactation period.

Material and Methods

Wistar rats were kept in a room with controlled temperature (25 ± 1°C) and with artificial dark-light cycle (lights on from 7:00 a.m. to 7:00 p.m.). Three-month-old, virgin female rats were caged with one male rat at a proportion of 2:1. After mating, each female was placed in an individual cage with free access to water and food until delivery. The female rats had a normal pregnancy, receiving food and water ad libitum until delivery. Then, the number of pups born was similar and all pups showed good health condition. Besides, no pups showed any statistical difference in body weight or linear growth. The use and handling of experimental animals followed the principles described in the Guide for the Care and Use of Laboratory Animals [17], and the study design was approved by the local Ethical Committee for the care and use of laboratory animals.

At delivery, six dams were each randomly assigned to one of the following groups: control group (C), with free access to a standard laboratory diet containing (in grams per 100 g) 23 protein, 66 carbohydrate, 11 fat, 17,038.7 total energy (kJ/kg); protein-restricted (PR) group, with free access to an isoenergy and protein-restricted diet containing 8% protein; energy-restricted group (ER), receiving a standard laboratory diet in restricted quantities, which were calculated according to the mean ingestion of the PR group. The protein-restricted diet was prepared in our laboratory by using the control diet (Nuvilab-Nuvital Ltd., Paraná, Brazil), with replacement of part of its protein content with cornstarch. The amount of the latter was calculated to replace the same energy content of the control diet. Vitamin and mineral mixtures were formulated to equal those found in the control diet and to meet the American Institute of Nutrition AIN-93G recommendation for rodent diets [18].

Within 24 h of birth, excess pups were removed so that only 6 pups were kept per dam, as it has been shown that this procedure maximizes lactation performance [19]. Malnutrition was started at birth, which was defined as day 0 of lactation (d0), and was ended at weaning (d21). To evaluate the nutritional state, the food consumption and body weight of the pups were monitored throughout the experiment.

At weaning, 6 rats were anesthetized with thiopental anesthesia (0.1 ml/100 g body weight) and perfused through the left ventricle with buffered saline followed by formalin solution. After perfusion, the testes were excised and weighed and post-fixed by immersion in the same fixative for 24 h at room temperature. Subsequently, the testes were paraffin-embedded, sectioned at 5-μm thickness and processed by routine histological methods. All samples were stained with hematoxylin and eosin.

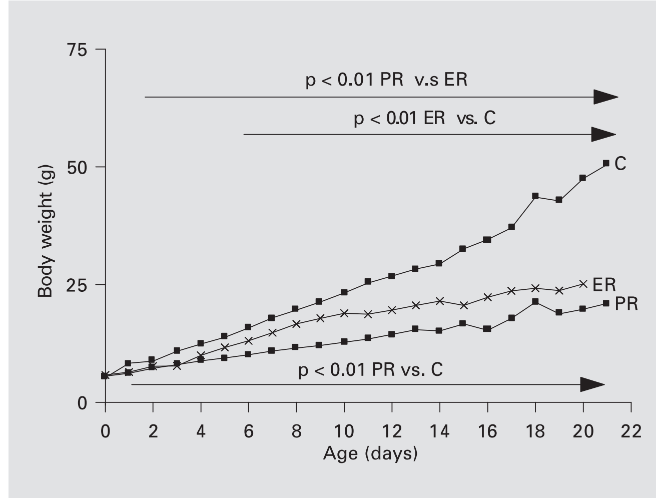

Fig. 1. Body weight in control group (C), protein-restricted group (PR), and energy-restricted group (ER). Values are given as mean ± SD of 6 animals.

From each section, five rounded tubules of five different random fields were evaluated, thus analyzing 25 test areas from each testis. The analyzed fields were digitized to a final magnification of 400× using a video camera coupled to a light microscope. In this study, we used the test system [20] that has uniform random test lines with a line separation (d). The number of intersections (I), between the profile boundary of seminiferous tubules and the test lines is counted and the perimeter is estimated according to the formula: $P = (\pi/2) \cdot d \cdot I$, where P = perimeter, d = distance between test lines, and I = intersection between test line and tubules border. The luminal and epithelial areas and epithelial height were calculated in micrometers.

The data were reported as mean ± SD. Statistical significance of experimental observations was determined by the Kruskal-Wallis test followed by the Mann-Whitney test. The level of significance was set at $p < 0.05$.

Results

The animals whose dams were submitted to protein or energy restriction in the lactation period presented a significant reduction ($p < 0.01$) in the body weight gain from day 2 until weaning. There was also a significant difference ($p < 0.01$) in the body weight of PR and ER groups from day 6 until weaning (fig. 1).

Table 1 depicts the rats weights, the testes weights, the luminal and epithelial areas and the epithelial height of the seminiferous tubules of the offspring at weaning. All parameters studied were significantly reduced in the PR and ER groups when compared to controls. The rats weights were significantly reduced in the PR compared to the ER group; we did not find any difference in the other parameters between these groups. Histological sections of the testes are shown in figure 2.

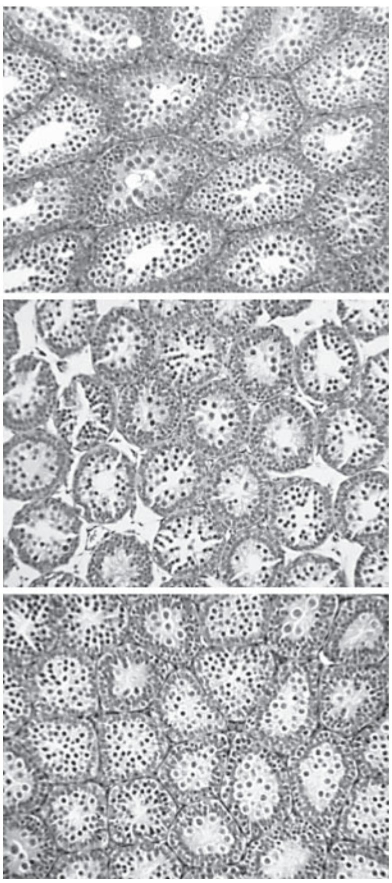
C
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Fig. 2. Histological sections of seminiferous tubules of the control (C), protein-restricted (PR), and energy-restricted (ER) groups. Final magnification ×200.

Discussion

Although the effects of undernutrition in the reproductive system of the rat is a well-documented phenomenon [15, 16], the consequences of maternal malnutrition during lactation in the testes of offspring appear to be novel. Therefore, the present study provides new insights into the effects of protein and energy restriction on the structural aspects of the testes in weaned rats.

In this paper, we showed that reduction in the protein/energy ratio of diets given to the lactating rats can markedly impair body and testes weights of offspring, and this finding is in agreement with the literature [13, 21–23].

We also showed that the effects of protein and energy-restricted diets on the structural characteristics of the testes are similar, since there was no significant difference in the histological parameters studied between PR and ER groups.

As we have shown recently [7], lactating rats given free access to a low-protein diet consumed only 60% of the diet when compared with the amount eaten by rats receiving the normal diet. Therefore, both restricted groups are being submitted to energy restriction, in spite of the fact that the PR diet has the lowest content of protein. This fact could suggest that the lack of energy could be more aggressive than the lack of protein and, for this reason, the structural alterations in both groups seem to be the same.

Malnutrition in the pre-pubertal period decreases body weight, testicular weight and prostatic weight [15]. Histological analysis of testes from pre-pubertal animals submitted to protein or energy restriction revealed a decrease in the diameter of the seminiferous tubules and in the size and number of Sertoli cells, as well as a delay in the functional maturing of Sertoli cells [15, 24]. Malnutrition during lactation seems to cause a similar disturbance in the testes of the offspring, since the testes weight and

Table 1. Testes weight, luminal and epithelial areas and epithelial height in the seminiferous tubules of testes of offspring whose dams were submitted to protein or energy restriction during lactation

	End of lactation (day 21)		
	control	protein-restricted	energy-restricted
Body weight, g	47.46 ± 2.26	19.98 ± 1.27*	25.38 ± 0.93*, #
Testes weight, g	0.1462 ± 0.018	0.0516 ± 0.0067*	0.058 ± 0.0026*
Total area, μm^2	12,678 ± 588.4	9,702 ± 504.8*	8,566 ± 406.6*
Luminal area, μm^2	2,988.5 ± 143.7	2,078 ± 107.4*	1,915.7 ± 105.2*
Epithelial area, μm^2	11,358 ± 577.2	9,014 ± 477.31*	8,232 ± 381.1*
Epithelial height, μm	69.65 ± 3.82	58.07 ± 1.58*	53.9 ± 1.67*

Values are given as the mean ± SEM of 6 animals. * $p < 0.01$ vs. C; # $p < 0.01$ vs. PR.

some structural aspects of seminiferous tubules were reduced in the rats testes at weaning.

The decrease observed in the structural aspects of seminiferous tubules of testes suggests that the nutritional status of the mother during lactation is essential to the normal growth and development of this organ. Indeed, rats undernourished during lactation present a delay in the onset of puberty [23, 25] and undernourishment during gestation and suckling in rats was reported to reduce fundamental parameters of both development and reproductive function in adult male offspring [26].

In this paper, we suggest that maternal malnutrition during lactation affects some structural characteristics of the testes in weaned rats that could alter the encoding of the organ and be responsible for future alterations in its physiology.

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