The pups’ endometrium morphology is affected by maternal malnutrition during suckling
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Received 22 July 2004; received in revised form 19 October 2004; accepted 21 October 2004

Abstract
Objectives: This study aims to determine the effects of maternal protein and energy malnutrition during lactation on the endometrial structures of the offspring at puberty.
Methods: At parturition, dams were randomly assigned to the following groups: control group (C), with free access to a standard laboratory diet containing 23% protein; protein-restricted (PR) group, with free access to an isonenergy and protein-restricted diet containing 8% protein; and energy-restricted (ER) group, receiving standard laboratory diet in restricted quantities. After weaning, all female pups had free access to standard laboratory diet. At puberty, the animals were sacrificed with pentobarbital and only females on the diestrum stage were used for the analyses. The stereological method used for quantifying the uterine endometrium was the M42 test system.
Results: When compared to C group, both PR and ER groups presented a significant reduction in the length density of the glands (PR = 53%, ER = 55.7%, p < 0.001), in the volumetric density of the epithelium (PR = 49%, ER = 38%, p < 0.001) and lumen (PR = 42.7%, p < 0.001; ER = 23.8%, p < 0.001) and in the surface density of the inner (PR = 22%, ER = 13.8%, p < 0.001) and outer (PR = 55.4%, p < 0.01; ER = 40.6%, p < 0.001) glands. The volumetric density of the stroma was significantly higher in both PR (114%, p < 0.001) and ER (117%, p < 0.001) groups. In all parameters studied, there was no significant difference between PR and ER groups.
Conclusions: Our results show that the protein and energy restriction during lactation leads to an atrophy of the uterine endometrial glands of the offspring at puberty.

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Keywords: Nutrition disorders; Lactation; Puberty; Endometrium; Morphology

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

Some authors showed that the nutritional status of the mother during gestational and lactational periods is essential to the normal growth and development in humans [3–5] or in experimental animals [6–10]. Also, some studies have shown that gestation and/or lactation could be a critical period for the future progeny’s nutritional and hormonal status [8,11–13], a relationship that has been termed metabolic imprinting. This term is defined by Waterland and Garza [14] as the basic biological phenomena that putatively underlie relations among nutritional experiences of early life and early diseases.

The offspring of rats submitted to malnutrition during lactation presents at weaning alterations in neural development [15], insulin secretion [16] and thyroid function [17]. It was showed either that mother’s nutrition during lactation could determine the body weight [17] and thyroid function [9,10] of her offspring in the adult life. Thus, lactation could be a critical period in determining the future endocrine status of the progeny.

Some authors showed that pre-pubertal malnutrition changes the serum and pituitary hormonal levels [18] morphological characteristics of gonadotrophs and mammotrophs [18], number of ovarian follicles [19], estrogen and progesterone receptors in the uterus [20,21] and the uterus collagen synthesis [22].

Recently, we have demonstrated that the female offspring whose mothers were submitted to protein and energy malnutrition during lactation, and had free access to a normal diet after weaning, presented at puberty a reduction in the body weight gain, linear growth, ovaries and uterus weight. Also, the onset of puberty was delayed, in spite of a normal estrous cycle [23]. Therefore, in the present study, we aim to evaluate, through a stereologic method, the effects of maternal protein and energy malnutrition during lactation on the uterine endometrial glands and stroma of the female offspring at puberty.

2. Methods

2.1. Protocol

Wistar rats were kept in a room with controlled temperature (25 ± 1 °C) and with artificial dark-light cycle (lights on from 7:00 am to 7:00 pm). 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 use and handling of experimental animals followed the principles described in the Guide for the Care and Use of Laboratory Animals [24] and the project was approved by the local Ethical Committee for care and use of laboratory animals.

Three dams were randomly assigned to one of the following groups: control group (C), with free access to a standard laboratory diet containing 23% protein; protein-restricted (PR) group, with free access to an iso-energy and protein-restricted diet containing 8% protein; and energy-restricted (ER) group, receiving standard laboratory diet containing 23% protein in restricted quantities, which were calculated according to the mean ingestion of the PR group. The low-protein diet was prepared in our laboratory and its composition is showed in Table 1. Vitamin and mineral mixtures were formulated to meet the American Institute of Nutrition AIN-93G recommendation for rodent diets [25].

<table>
<thead>
<tr>
<th>Ingredient (g/kg)</th>
<th>Control</th>
<th>Protein-restricted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein (g/kg)</td>
<td>23.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Corn starch (g/kg)</td>
<td>676.0</td>
<td>826.0</td>
</tr>
<tr>
<td>Soybean oil (g/kg)</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Vitamin mix (g/kg)</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Mineral mix (g/kg)</td>
<td>40.0</td>
<td>40.0</td>
</tr>
</tbody>
</table>

Macronutrient composition (%):
- Protein: 23.0 vs 8.0
- Carbohydrate: 66.0 vs 81.0
- Fat: 11.0 vs 11.0

Total energy (kJ/kg): 17038.7 vs 17038.7

a The principal protein resources are soybean, wheat, steak, fish and amino acids.
b Standard diet for rats (Nuvilab-Cristal Ltd., Paraná, Brazil).
c The protein-restricted diet was prepared in our laboratory by using the control diet, 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.
d Vitamin and mineral mixtures were formulated to meet the American Institute of Nutrition AIN-93G recommendation for rodent diets [25].
Within 24 h of birth, excess pups were removed so that only six female pups were kept per dam, as it has been shown that this procedure maximizes lactation performance [26]. Malnutrition of the studied rats was started at birth, which was defined as day 0 of lactation (d0), and was ended at weaning (d21). After weaning, the female pups of the same treatment group were housed in groups of three animals per cage, and given unlimited access to food and water until puberty (day 40), when the animals were anesthetized and sacrificed with a lethal dose of pentobarbital.

To evaluate the nutritional state, we monitored food consumption, body weight and linear growth (no–se–tail) throughout the experiment (data not shown). At the slaughtered, cyclic stages of the ovaries were studied by vaginal smears and only rats on the diestrus stage were used for the analyses. The uterus was carefully removed, weighed, and fragments were obtained according to the vertical sections method [27]. The M42 multipurpose test system was used where only well-preserved structures were used for the analyses. The uterus was considered. The short-line length (d) was used to calibrate the test system, the line length L is 21d, the test area AT is 36.36d², and it has 42 test points (Pt). The stereological methods were described in detail elsewhere.

The stereological parameters analyzed were: volumetric density (Vv) of the endometrial stroma; (2) volumetric density (Vv) of the glandular epithelium; (3) volumetric density (Vv) of the glandular lumen; (4) surface density (Ss) of the inner and outer glands (Ss = 2L/At, where L are intersections of the inner and outer glandular surfaces with the test line and At is the length of test line); (5) length density (Lv) of the glands (Lv = 2QA (mm/mm²), where QA is the number of the glandular profiles in the test area).

### 2.3. Statistical analysis

The data was reported as mean ± standard deviation. Statistical significance of experimental observations was determined by the Kruskal–Wallis test followed by Dunn’s multiple comparison test. The level of significance was set at p < 0.05.

### 3. Results

The volumetric density of the glands (lumen and epithelium) is shown in Fig. 1. All these parameters were significantly reduced in both malnourished groups (p < 0.001) compared to the C group, and there was no difference between the PR and ER groups.

The Vv of the epithelium was significantly lower in both PR (49%, p < 0.001) and ER (38%, p < 0.001) groups when compared to the control group (Fig. 1b). The Vv of the lumen area was significantly lower in both PR (42.7%, p < 0.001) and ER (23.8%, p < 0.001) groups when compared to the control group (Fig. 1a).

The Ss of the inner glands was significantly lower in both PR (22%, p < 0.001) and ER (13.8%, p < 0.001) groups, when compared to the control group (Fig. 1c).

The Ss of the outer glands was significantly lower in both PR (55.4%, p < 0.01) and ER (40.6%, p < 0.001) groups when compared to the control group (Fig. 1d).

The volumetric density of the stroma was significantly higher in both PR (114%, p < 0.001) and ER (117%, p < 0.001) groups when compared to the C group (Fig. 2).

The length density of the glands was significantly lower in both PR (53%, p < 0.001) and ER (35.7%, p < 0.001) groups when compared to the C group (Fig. 3).

The histological sections of uterus are shown in the Fig. 4.
4. Discussion

The concept of programming associates physiological changes in adulthood with determined physiological changes in gestational or neonatal period [32]. Chronic diseases in the adulthood, as coronary insufficiency and the pharnmetabolic syndrome (diabetes mellitus, hypertension and obesity) could be programmed in the initial stages of life [33]. Previous studies showed that maternal undernutrition during lactation causes alterations in the milk composition [8], body weight [17], thyroid function [9,10] and leptin serum concentration of pups at weaning [34]. When those animals reach the adulthood, the alterations in...
body weight and thyroid function are persistent [35], reinforcing the metabolic imprinting concept. Protein and energy restriction during lactation also leads to growth retardation, reduction in the ovaries and uterus weights and delayed on the puberty onset in pups [23].

The low gain in body weight observed in the undernourished female offspring could be due to lack of growth hormone (GH) of effects, since some authors described that food deprivation decreased GH-releasing hormone (GHRH) [36,37].

The morphometric results show that both groups on dietary restriction present a significant reduction in the number and lobular aspect of the endometrial glands, with a compensatory increment in the stroma, suggesting a glandular atrophy at puberty. As we know that endometrial development is dependent of gonadal hormones, lack of these hormones effects could result in endometrial atrophy.

The protein and energy malnutrition can alter the hypothalamo-hypophyseal-gonadal axis. It is clear that the GnRH pulse generator system and gonadotrophin release are inhibited by undernutrition [18,38–40] and that undernutrition causes morphological alterations in the mammotrophs and gonadotrophs that are typical of those cells whose secretory activities are suppressed [18,41]. Quantitative immunohistochemistry revealed a decrease in the number of gonadotrophs of undernourished adult monkeys that can be correlated with ultrastructural findings [37].

The delay on the onset of puberty observed in these animals, caused by maternal malnutrition [23], is probably due to an incomplete mature state of the hypothalamic-pituitary-gonadal axis. So, the low-hormonal stimulus could be insufficient to cause the normal development of the endometrial glands, and, probably, the uterus could not be mature enough to respond to the hormonal stimulus. This speculation could explain the atrophy of the endometrial glands.

Leptin, an adipocyte-secreted plasma hormone involved in the control of food intake and energy expenditure that plays a key role in body-weight homeostasis, has recently emerged as a pivotal signal in the regulation of fertility and in the control of pituitary gonadotrophins—LH and FSH [42]. It was related that energy restriction during lactation causes a drastic reduction in plasma leptin level in the weaned female offspring [43]. In vitro studies have shown that GH stimulates mitogenesis and amplifies the effects of FSH on the induction of LH receptors and on steroidogenesis by granulosa cells in numerous species [44]. So, the low leptin levels could contribute to alterations on
the hypothalamic-hypophyseal-gonadal axis, decreasing LH and FSH levels resulting in low estradiol serum levels.

Some authors showed that uterus’ weight as well as estrogen and progesterone receptors are reduced in the uterus of protein and energy restricted pregnant and non-pregnant rats [20,21]. As these hormones are responsible for growing and development of endometrial glands, it is possible that lack of these receptors could lead to the reduction observed in the number and lobular aspect of endometrial glands in undernourished pups.

Although these authors have described that malnutrition leads to a state of hypofunction of the hypothalamic-pituitary-gonadal axis, the majority of them evaluated the effects of malnutrition in male animals. Besides, some authors described that in female rats, malnutrition during lactation time induces a drastic reduction of plasma leptin without affecting the circulating levels of LH, estradiol and progesterone [43]. Also, Abeceia et al. [45] showed that the mean content of progesterone in the endometrium, the progesterone concentration in the ovarian vein, and the production of progesterone by corpus luteum are not affected by nutritional state. These data reinforce the hypothesis that the uterus is immature to respond to hormonal stimulus, which could explain the atrophy of the endometrial glands.

Until this moment, we do not have knowledge on how malnutrition can affects the expression of estrogen, progesterone and androgen receptors in the uterus. Estrogen appears as the major regulator of estrogen function in the uterus and Erβ can modulate Erα activity in a response-specific and dose-dependent manner [46]. Estrogen increases progesterone receptor expression specifically in uterine stromal cells of the oERKO mouse, suggesting that Erβ may be capable of mediating some of estrogen effects in the uterus [47]. Although full response of the epithelium is dependent on an Erα-positive mesenchyme, stromal cell proliferation is independent of tissue Erα [48]. Also, diethylstilbestrol stimulates uterine progesterone expression in the glandular cell, but not in the luminal epithelial cells [49]. These results can probably explain our data of increase in volumetric density of stroma in face of reduction in glandular parameters.

Androgen can also be implicated in the uterine derangement responsiveness caused by maternal malnutrition during lactation since it was demonstrated that in the uterus, besides in other organs, there is nuclear staining for androgen receptors in glandular and luminal epithelial cells and in the stroma in which glands are embedded [50,51].

In conclusion, our results show that protein and energy restriction during lactation leads to an atrophy of the uterine endometrial glands in the offspring at puberty.

Acknowledgements

Supported by grants from the National Council of Scientific and Technological Development (CNPq), Foundation for Research Support of Rio de Janeiro (FAPERJ), and Coordination for Improvement of Post-Graduated Students (CAPES), Brazil.

References


