

# Puberty onset in the female offspring of rats submitted to protein or energy restricted diet during lactation

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## Abstract

This study aims to determine the effects of maternal protein and energy malnutrition during lactation on the linear growth, body weight and onset of puberty of the female offspring. At parturition, dams were randomly assigned to the following groups: (C) control group, with free access to a standard laboratory diet containing 23% protein; (PR) protein-restricted group, with free access to an isoenergy and protein-restricted diet containing 8% protein; and (ER) energy-restricted group, receiving standard laboratory diet in restricted quantities. After weaning, the female pups had free access to standard laboratory diet. From day 30 onwards, the pups were inspected daily for vaginal opening. Cyclic stages of the ovaries were studied by daily vaginal smears after vaginal opening until day 40 when all animals were sacrificed with pentobarbital. From day 4 after birth until day 40, body weight and linear growth in the PR and ER rats were significantly lower than in controls ( $p < 0.001$ ). In spite of the significant ( $p < 0.05$ ) delayed in the vaginal opening in PR and ER rats, the first estrous cycle occurred at the same time of vaginal opening in all groups. The PR and ER rats exhibited a lower uterine (PR = 42%, ER = 40%,  $p < 0.001$ ) and ovarian (PR = 26%, ER = 19%,  $p < 0.05$ ) absolute weight and uterus relative weight (PR = 27%, ER = 22%,  $p < 0.05$ ). Our data showed that maternal protein and energy malnutrition during lactation leads to growth retardation and delayed on the onset of puberty in female pups, with vaginal opening and estrous cycle occurring at the same time. © 2004 Elsevier Inc. All rights reserved.

*Keywords:* Nutrition disorders; Lactation; Puberty; Body weight; Rats

## 1. Introduction

Puberty can be defined as a maturational process of the hypothalamic-pituitary-gonadal axis, which starts during the fetal period and reaches a completely mature state in adolescence. The pubertal development starts soon after weaning, and the vaginal opening is the first visual sign during this development, being commonly used in literature to indicate the onset of puberty.

In the female rat, vaginal opening and ovulation take place when the hypothalamic-pituitary-ovarian axis becomes fully mature [1]. In normal female rats, the estrous cycle and ovulation occur at the same time as vaginal opening occurs [2,3].

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The prenatal and early postnatal nutritional status plays a critical role in postnatal growth and development. Early malnutrition may change the original programming of organs, especially those in developmental phases, which can result in metabolism long-term changes [4–6].

For a long time it has been hypothesized that weight plays an important role in the central regulation of puberty. Some authors proposed a direct connection between body size and sexual maturation, suggesting that menarche is triggered by the attainment of a particular body weight [7,8]. Subsequently, this idea has been modified to the critical body fat hypothesis, which proposes that menarche is triggered by attainment of a particular percentage of body fat [9,10]. However, more recent studies, in humans [11,12] and animals [2,3,13–16] show that a critical body weight does not seem to be essential to the onset of puberty.

Intrauterine growth retardation results in a delayed in the onset of puberty in female rats, in contrast to postnatal food restriction, which did not alter the onset of puberty [3,13,14]. In addition, intrauterine growth-retarded rats have a significantly lower number of follicles in the ovaries at

vaginal opening, whereas postnatally food restricted rats have a normal number of follicles, but impaired follicle maturation. Growth-retarded groups did not ovulate at onset of puberty, in contrast to control rats, which exhibited ovulation and vaginal opening at the same time [15].

These findings indicate that the perinatal period appears to be a critical period for the maturational process of sexual development in the rat, and that malnutrition during different periods may affect ovarian development in different ways.

The aim of this study was to determine the effects of maternal protein and energy malnutrition during lactation on the linear growth, body weight and onset of puberty of the female offspring.

## 2. Methods and materials

**Protocol:** Wistar rats were kept in a room with controlled temperature ( $25 \pm 1^\circ\text{C}$ ) 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 use and handling of experimental animals followed the principles described in the Guide for the Care and Use of Laboratory Animals [17], and the project was approved by the local Ethical Committee for the care and use of laboratory animals.

Nine dams were each randomly assigned to one of the following groups: (C) control group, with free access to a standard laboratory diet containing 23% protein; (PR) protein-restricted group, with free access to an isoenergy and protein-restricted diet containing 8% protein; and (ER) energy-restricted group, receiving standard laboratory diet 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 [18].

Within 24 hr of birth, excess pups were removed so that only 6 female 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). After weaning, the female pups of the same treatment group were housed in group of three animals per cage, and given unlimited access to food and water.

**Measurements:** To evaluate the nutritional state, the food consumption, body weight and linear growth (nose-tail) were monitored throughout the experiment. From day 30 onwards, the females were inspected daily for vaginal opening (VO). Onset of puberty was defined as the age (in days) in which VO occurred. Cyclic stages of the ovaries were studied by daily vaginal smears after vaginal opening until

Table 1  
Composition of control and protein-restricted diets

	Control <sup>§</sup>	Protein-Restricted*
Ingredients (g/Kg)		
Total protein <sup>♣</sup>	230.0	80.0
Corn starch	676.0	826.0
Soybean oil	50.0	50.0
Vitamin mix <sup>†</sup>	4.0	4.0
Mineral mix <sup>†</sup>	40.0	40.0
Macronutrient composition (%)		
Protein	23.0	8.0
Carbohydrate	66.0	81.0
Fat	11.0	11.0
Total energy (KJ/Kg)	17038.7	17038.7

<sup>♣</sup> The principle protein resources are soybean wheat, steak, fish and amino acids.

<sup>§</sup> = Standard diet for rats (Nuvilab-Nuvital Ltd., Paraná, Brazil).

\* The protein-restricted diet was prepared in our laboratory by using the control diet, with replacement of part of its protein content with corn starch. The amount of the latter was calculated to replace the same energy content of the control diet.

<sup>†</sup> Vitamin and mineral mixtures were formulated to meet the American Institute of Nutrition AIN-93G recommendation for rodent diets [18].

day 40 when all animals were anesthetized, had blood collected by cardiac puncture and were sacrificed with a lethal dose of pentobarbital. The uterus and ovaries were then dissected and weighted.

Blood samples were centrifuged to obtain serum, which was individually kept at  $-20^\circ\text{C}$  until assay. Serum albumin was used as an index of protein malnutrition and was determined by a colorimetric method (Bioclin, Belo Horizonte, MG, Brazil).

**Data analysis:** The data was reported as mean  $\pm$  standard deviation. Statistical significance of experimental observations was determined by the one-way analysis of variance followed by Newman Keuls test [20]. The level of significance was set at  $P < 0.05$ .

## 3. Results

The body weight and linear growth characteristics of protein-restricted (PR), energy-restricted (ER) and control (C) female pups at days 0, 21 and 40 are shown in Fig. 1. Both malnourished groups presented a significant ( $P < 0.001$ ) lower body weight compared to the control group at day 21 (PR = 53%, ER = 42%) and day 40 (PR = 21%, ER = 19%), (Fig. 1A). A similar significant reduction ( $P < 0.001$ ) was observed in the linear growth of the malnourished groups at day 21 (PR = 26%, ER = 19%) and day 40 (PR = 11%, ER = 11%), (Fig. 1B). Also, both the body weight and linear growth were significantly lower in the PR group compared to the ER group at day 21 ( $P < 0.05$ ), (Fig. 1A and 1B).

Overall, at each time point of measurement from day 4

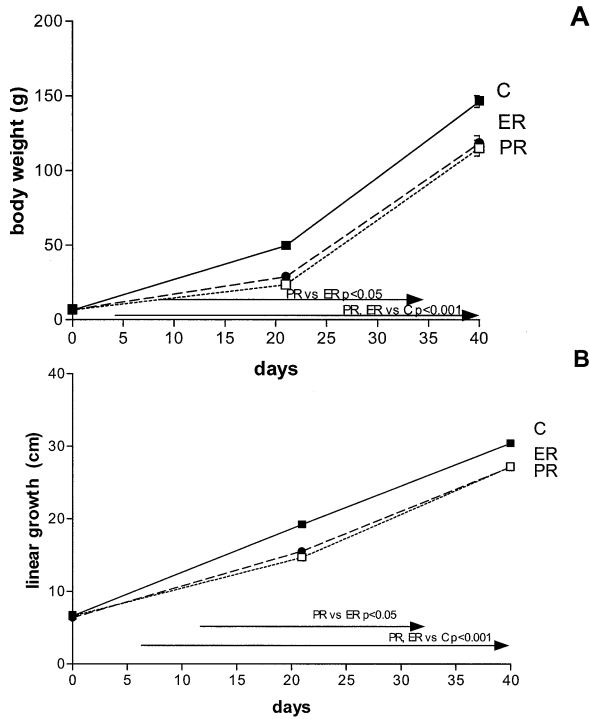


Fig. 1. Body weight (1A) and linear growth (nose-tail) (1B) in control group (C), protein-restricted group (PR), and energy-restricted group (ER). Values are given as mean  $\pm$  standard deviation of 14 animals.

after birth until day 40, body weight in the PR and ER groups were significantly lower when compared to controls ( $P < 0.001$ ). In addition, the body weight of the PR groups were significantly lower ( $P < 0.05$ ) than ER from day 8 until day 35 (Fig. 1A). Similarly, the linear growth showed the same pattern, that is, the PR and ER groups had significantly lower linear growth ( $P < 0.001$ ) when compared to controls from day 6 after birth until day 40. Also, the linear growth of the PR group was significantly lower ( $P < 0.05$ ) than the ER group from day 12 after birth until day 32 (Fig. 1B).

The day of vaginal opening was significantly delayed in PR and ER groups, when compared to controls ( $C = 30.2 \pm 0.5$ ;  $PR = 33.5 \pm 0.9$ ;  $ER = 32.6 \pm 0.8$ ,  $p < 0.05$ ). In all groups, the first estrous cycle occurred at the same time of vaginal opening.

At the time the animals were killed the PR and ER groups exhibited a significantly lower uterine ( $PR = 42\%$ ,  $ER = 40\%$ ,  $P < 0.001$ ) and ovarian ( $PR = 26\%$ ,  $ER = 19\%$ ,  $P < 0.05$ ) absolute weights than those of controls. However, after adjusting for body weight, the uterine weight was still significantly lower ( $PR=27\%$ ,  $ER=22\%$ ,  $P < 0.05$ ), but the ovaries weight did not show significant alteration (Fig. 2). The albumin serum concentration did not show any significant difference among the groups ( $C = 3.6 \pm 0.23$ ;  $PR = 3.6 \pm 0.06$ ;  $ER = 4.1 \pm 0.11$ ).

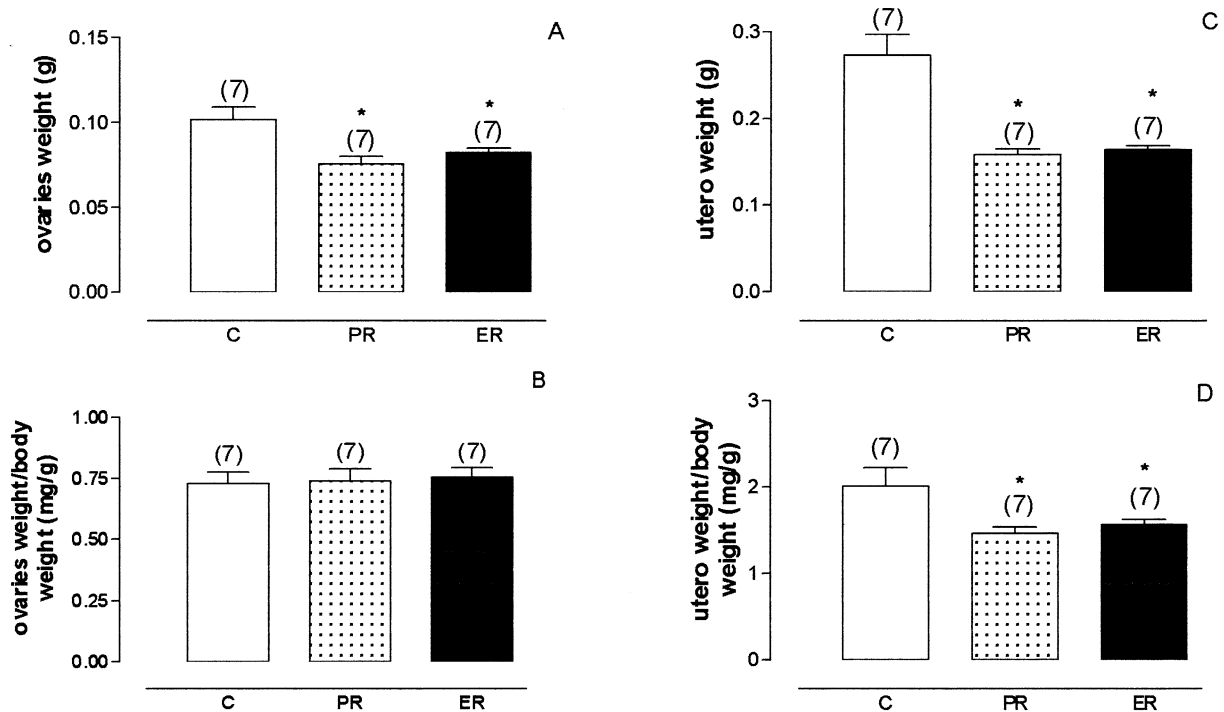


Fig. 2. Absolute and relative weights of ovaries (A, B) and uterus (C, D) of pups in control group (C), protein-restricted group (PR), and energy-restricted group (ER) at 40 days old. Values are given as mean  $\pm$  standard deviation. The number of animals studied is shown in parentheses.  $P < 0.05$  compared with control group.

#### 4. Discussion

Numerous data from various studies in humans and animals have shown that early malnutrition can influence postnatal growth and development, changing the original programming of organs, especially those in developmental phases, and resulting in long-term changes in metabolism [4–6].

In this study we showed that maternal malnutrition during the lactation period causes a reduction in the growth and a delayed onset of puberty of both protein and energy restricted pups. These data are partially in agreement with the literature. Many studies showed that intrauterine or postnatal malnutrition led to a growth retardation in the pups [2,3,13–16], but only intra-uterine malnutrition resulted in a delay in the onset of puberty in female rats, in contrast to postnatal food restriction, which did not influence the onset of puberty [3,13,14]. Despite the differences in the effects of postnatal malnutrition in the onset of puberty, all studies agree that the onset of puberty does not depend on the achievement of a certain crucial weight.

We also showed that maternal malnutrition did not alter the estrous cycle, since all pups exhibited ovulation and vaginal opening at the same time. In contrast, Engelbregt et al. [15] have shown that both intrauterine and postnatal growth-retarded groups did not ovulate at the onset of puberty. Keisler et al. [21] showed that pre pubertal animals would not enter puberty until they are well fed and that normally cyclic females will stop cycling when submitted to extreme malnutrition. The fact that the PR and ER groups had free access to food from weaning until sacrifice, besides the normal albumin serum levels, suggests that in spite of the lower body weight, the animals were not malnourished at that time. Therefore, it seems that the onset of puberty is more severely affected by the nutrition state than the estrous cycle.

The decreased observed in the relative weight of the uterus suggests that this organ can be affected directly by maternal malnutrition in contrast to the ovaries that shown a normal relative weight in both malnourished groups. Studies are in progress in our laboratory to evaluate the morphological aspects of these organs, the capability of the uterus to maintain a normal pregnancy, and the number of ovarian follicles, as well as its maturation state.

Many of those studies cited previously evaluated the effects of malnutrition during pregnancy by ligation of the uterine artery on day 17 of gestation, or during lactation by litter-enlargement to 20 pups per mother. In our present study, malnutrition was achieved by offering the dams 2 different kinds of diet, a protein-restricted diet or an energy-restricted diet. We have showed recently that the milk composition from those dams submitted to protein or energy restriction is altered, presenting a significant reduction in the protein content in the PR group, and a significant increment in the protein and lipids content in the ER group [22].

Therefore, the perinatal period appears to be a critical

time for the maturational process of pubertal development, nevertheless, malnutrition in this period can cause different results, which could be explained by the use of different strains of rats, different diets, and different ways to get malnutrition, which can result in different grades of malnutrition.

In conclusion, our data shows that maternal protein and energy malnutrition during lactation leads to a growth retardation and a delayed onset of puberty in female pups with vaginal opening and estrous cycle occurring at the same time.

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