Effects of maternal undernutrition during lactation on aromatase, estrogen, and androgen receptors expression in rat testis at weaning

Cíntia Vilanova Teixeira, Dorothée Silandre[1], Alba Marcelly de Souza Santos, Christelle Delalande[1], Francisco J B Sampaio, Serge Carreau[1] and **Cristiane da Fonte Ramos**

Urogenital Research Unit, State University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil

[1]Biochemistry, INRA2006-EA2608, University, Esplanade de la Paix, 14032, CAEN-Cedex, France

(Requests for offprints should be addressed to S Carreau; Email: serge.carreau@unicaen.fr)

(C da Fonte Ramos is in US postdoctoral position at Stanford University, Department of Urology, California, USA).

Abstract

The goal of this study was to evaluate the effects of maternal malnutrition during lactation on serum levels of testosterone and estradiol, testicular testosterone concentration, aromatase, testicular androgen (AR) and estrogen α (ERα) receptors expression in the pups at weaning. From parturition until weaning, Wistar rats were separated into three groups: (C) control group, with free access to a standard laboratory diet containing 23% protein; protein-energy restricted (PER) group, with free access to an isoenergy and protein-restricted diet containing 8% protein; and energy-restricted (ER) group, receiving standard laboratory diet in restricted quantities, which were calculated according to the mean ingestion of the PER group. All pups were killed at weaning, corresponding to 21 days *post partum*. Compared with the C group, body weights ($C=48\pm2{\cdot}3$ g; $PER=20\pm1{\cdot}3$ g; $ER=25{\cdot}4\pm0{\cdot}9$ g; $P<0{\cdot}01$) and testicular weights ($C=0{\cdot}15\pm0{\cdot}02$ g, $PER=0{\cdot}05\pm0{\cdot}01$ g, $ER=0{\cdot}06\pm0{\cdot}02$ g, $P<0{\cdot}001$) of both PER and ER groups were lower. However, there was no significant difference in the testicular/body weight ratio in PER and ER groups compared with the C group. The testosterone serum concentration (ng/ml) was significantly higher in the PER group compared with ER and C groups ($C=0{\cdot}09\pm0{\cdot}012$; $PER=0{\cdot}45\pm0{\cdot}04$; $ER=0{\cdot}15\pm0{\cdot}03$, $P<0{\cdot}01$). Testicular testosterone concentration ($C=2{\cdot}1\pm0{\cdot}43$; $PER=6{\cdot}5\pm0{\cdot}7$; $ER=13\pm2{\cdot}3$, $P<0{\cdot}01$) was increased in treated groups when compared with controls. The estradiol serum concentration (pg/ml) was lower in both dietary groups ($C=74\pm4{\cdot}6$; $PER=49\pm3{\cdot}2$; $ER=60\pm5{\cdot}5$, $P<0{\cdot}01$). The amounts of aromatase mRNA and ERα transcripts were significantly lower ($P<0{\cdot}05$) in PER and ER groups; conversely AR (both mRNA and protein) was significantly enhanced ($P<0{\cdot}05$) in treated animals. The nutritional state in early phases of development is important since we have demonstrated here that the maternal malnutrition during lactation leads to alterations in estradiol and testosterone serum concentrations, testicular testosterone concentration, AR and ERα expression together with a decrease of aromatase expression. All together, these changes of steroid status may be deleterious for future germ cell development and reproductive function of these male pups submitted to early malnutrition.

Journal of Endocrinology (2007) **192,** 301–311

Introduction

Although testosterone is recognized as the main sexual steroid in the male, estrogens are also produced in the testis and their concentrations within the male reproductive tract are generally higher than in the general circulation (Hess 2000). During the past years, accumulating evidence suggests that estrogens can play a physiological role in male reproduction (Sharpe 1998, Carreau *et al.* 1999, 2003, Simpson *et al.* 2000). The ability of the testis to transform androgens into estrogens is related to the presence of a microsomal enzymatic complex called aromatase (Simpson *et al.* 1994). However, even though the presence of aromatase and estrogen receptors is demonstrated in testicular cells, the role of estrogens in male reproduction is not fully understood and thus has not been extensively studied (Lambard *et al.* 2005).

Androgen (AR) and estrogen (ERα, ERβ) receptors are members of the steroid/thyroid hormone receptor gene superfamily and, like other steroid receptors, are mainly nuclear and function as ligand-dependent transcription factors (Lindzey *et al.* 1994, Zhou *et al.* 1994). Using testicular cell cultures, Cardone *et al.* (1998) showed that both estrogen and androgen may autoregulate the expression of their own receptor mRNAs, yet estrogen can downregulate AR mRNA.

Androgens can promote downregulation of AR mRNA concentrations (Quarmby *et al.* 1990, Krongrad *et al.* 1991, Hackenberg *et al.* 1992, Wolf *et al.* 1993), although androgenic

DOI: 10.1677/joe.1.06712
0022–0795/07/0192–301
Online version via http://www.endocrinology-journals.org

upregulation of receptor mRNA has been observed in few tissues (Gonzalez-Cadavid *et al.* 1993, Nastiuk & Clayton 1994, Kerr *et al.* 1995). The removal of endogenous androgens by ethane dimethane sulfonate treatment of adult rats, resulted in an obvious decrease in the AR immunoexpression, but replacement of testosterone for 4 h restored the expression of this receptor (Turner *et al.* 2001). The physiological significance of AR autoregulation has not been established; however, cellular AR concentration correlates relatively well with the extent of androgen responsiveness, suggesting that autoregulation of AR may influence hormonal sensitivity (Dai *et al.* 1996). There is also evidence showing that estradiol regulates *ER* gene expression in a dose-dependent manner (Tena-Sempere *et al.* 2000).

Undernutrition is known to have a wide variety of effects on endocrine systems (Rocha de Melo & Guedes 1997, Ramos *et al.* 2000, Cónsole *et al.* 2001). Regarding the reproductive system, it has been shown that food restriction can inhibit both the maintenance and onset of reproductive capability (Desjardins & Lopez 1983). However, long-term caloric restriction can transiently suppress the decreases of steroidogenic activities that are characteristics of aging (Chen *et al.* 2005).

In adult rats, food restriction can reduce the body weight (Chik *et al.* 1989), as well as testes, epididymis, and prostate weights (Grewal *et al.* 1971). The serum concentration of luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosterone are also decreased by undernutrition (Grewal *et al.* 1971, Howland 1975, Herbert 1980, Chik *et al.* 1987, 1989, Dong *et al.* 1994, Young *et al.* 2000). It was shown that malnutrition led to gonadotroph morphological alterations that are typical of those found in cells whose secretory activities are suppressed (Herbert 1980).

Despite the fact that undernutrition-related reproductive suppression in rats is a well-documented phenomenon, we do not have preceding knowledge until now, on how undernutrition affects aromatase and steroid receptor (AR and ERs) expressions in testicular tissues of male rats whose mothers were subjected to undernutrition during lactation, as well as on estradiol and testosterone serum concentrations. Therefore, the present study was designed to identify underlying mechanisms related to potential reproductive problems associated with undernutrition during the neonatal period.

Materials and Methods

Animals

Wistar rats were kept in a room with controlled temperature (25 ± 1 °C) and an artificial dark–light cycle (lights on from 0700 to 1900 h). Virgin female rats of 3 months of age were caged with one male rat at a proportion of 2:1. After mating, determined by the presence of a vaginal plug, each female was placed in an individual cage with free access to water and food until delivery. The handling of the animals was approved by the Animal Care and Use Committee of the Biology Institute of State University of Rio de Janeiro, which based their analysis on the Guide for the Care and Use of Laboratory Animals (Bayne 1996), and the study design was approved by the local Ethical Committee for the care and use of laboratory animals.

Experimental design

Six pregnant Wistar rats were separated after delivery into three groups: (C) control group – with free access to a standard laboratory diet containing 23% protein; protein-energy restricted (PER) group – with free access to an isoenergy and protein-restricted diet containing 8% protein; and energy-restricted (ER) group – receiving standard laboratory diet in restricted quantities, which were calculated, every day, according to the mean ingestion of the PER group and correspond to 60% of that consumed by the control group. The PER group, in spite of having free access to diet, consume about 60% of that consumed by the control group (Passos *et al.* 2000). In this way, the amount of food consumed in both ER and PER groups was almost the same and was measured every day (data not shown).

Within 24 h of birth, excess pups were removed, so that only six pups were kept per dam, because it has been shown that this procedure maximizes lactation performance (Fishbeck & Rasmussen 1987). At weaning (21 days *post partum*), seven male pups of each group were slaughtered under thiopental anesthesia (0·10 ml/100 g body weight), always in the morning. The blood was collected by cardiac puncture and the serum kept at -20 °C for subsequent determination of hormonal parameters. The testes were excised, dissected, weighted then divided in two parts: one was kept at -70 °C for subsequent measurements of aromatase, estrogen, and AR transcripts by RT-PCR and proteins by western blotting as well as endogenous testosterone by RIA. The other part of the testes was paraffin-embedded, sectioned at 5 μm thickness and processed by routine histological analyses. Some samples were stained with hematoxylin and eosin to check the integrity of the tissue, while others were used for AR and ERα immunohistochemistry.

Nutritional diet

The low-protein diet was prepared in our laboratory and its composition is shown in Table 1. Vitamins and mineral mixtures were formulated to meet the American Institute of Nutrition AIN-93G recommendation for rodent diets (Reeves *et al.* 1993).

RNA extractions

Total RNA from testicular tissue was extracted using the guanidium thiocyanate–phenol–chloroform method (Chomczynski & Sacchi 1987). Testes were homogenized in a lysis buffer (1 M Tris, 4 M guanidium thiocyanate, 0·5% sarcosyl, 1% β-mercaptoethanol). Then, RNA was extracted by a phenol/chloroform/isoamylic alcohol solution and precipitated by isopropyl alcohol. After washing with 75%

Table 1 Composition of control and protein-restricted diets

	Control[a]	Protein-restricted[b]
Ingredients (g/kg)		
Soybean+Amino acids	230·0	80·0
Corn starch	676·0	826·0
Soybean oil	50·0	50·0
Vitamin mix[c]	4·0	4·0
Mineral mix[c]	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)	17 038·7	17 038·7

[a]Standard diet for rats (Nuvilab-Nuvital Ltd, Paraná, Brazil).
[b]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.
[c]Vitamin and mineral mixtures were formulated to meet the American Institute of Nutrition AIN-93G recommendation for rodent diets (Reeves *et al.* 1993).

ethanol, the RNA was dried and dissolved with diethyl pyrocarbonate-treated water. The quality of RNA samples was verified by determination of the ratio 260 nm/280 nm and electrophoresis on a 1% agarose gel. The samples were stored at −80 °C until utilization.

Semi-quantitative RT-PCR

Total RNA (2 μg) was reverse transcribed into cDNA in a total volume of 40 μl. RNA was incubated at 42 °C for 1 h with 200 IU Moloney murine leukemia virus reverse transcriptase (M-MLV-RT), 0·5 mM dNTP, 0·2 μg oligo-dT and 20 IU Rnasin. Then, cDNA coding for aromatase, AR, estrogen receptors α and β and ribosomal protein L19 were amplified by PCR with specific primers (Table 2). The reactions were performed in a final volume of 50 μl from 4 μl cDNA with 1·5 IU Taq polymerase, 0·2 mM dNTP, 1·5 mM $MgCl_2$ and 25 pmoles of the forward and reverse primers. Primers were obtained from Invitrogen and all others products used for RT-PCR are from Promega. In order to quantify aromatase, ERα and AR transcripts, we have determined the optimal number of amplification cycles, for each gene. After an initial step of denaturation at 95 °C for 5 min, a variable number of cycles of amplification: 30 s at 95 °C, 30 s at 60 °C, 45 s at 72 °C were performed (Table 2), followed by a final extension at 72 °C for 7 min. We have chosen L19 transcripts which do not vary among all the samples in order to correct the difference in the quantities of total RNA used for reverse transcription (Tena-Sempere *et al.* 2002). All cDNA fragments amplified were run on a 2% agarose gel stained with ethidium bromide, visualized under u.v. transillumination and analyzed with NIH software (http://rsb.info.nih.gov/nih-image). For each cDNA amplified, we have verified their identity by sequencing (GENOME express, Meylan, France) and alignment with the published sequences (Table 2).

Extraction of proteins and western blotting

Each testis was homogenized in 500 μl TEG buffer (50 mM Tris pH 7·4, 1·5 mM EDTA, 50 mM NaCl, glycerol 10%, 5 mM DTT, 10 μg/ml leupeptin). The homogenate was centrifuged at 100 000 ***g*** for 2 h at 4 °C. The proteins extracted from the supernatants were measured by the Bradford method (Bradford 1976) and subsequent quantification of estrogen and AR was determined by western blot analysis. Total protein (70 μg) was loaded in each lane of 8% PAGE. The proteins were transferred to nitrocellulose membrane, and the detection of specific proteins was done with AR-specific antibody (rabbit polyclonal 200 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ERα-specific antibody (mouse monoclonal 200 μg/ml, Santa Cruz Biotechnology). Horseradish peroxidase IgG was used as a secondary antibody followed by autoradiography using enhanced chemic luminescence (ECL) detection reagents supplied by Amersham (Braunschweig, Germany). Bands were scanned in a computer and their relative intensities determined by densitometry using Scion Image v. 4.0.2 (Scion Corporation, Frederick, USA). The molecular weights of AR and ERα have been determined using a molecular weight standard kit.

Table 2 Oligonucleotide sequences used for amplification and cycling conditions

	Primer	Sequence (5′–3′)	Size of product (bp)	PCR cycles
Gene				
Aromatase (M33986)	5′ARO	GCTTCTCATCGCAGAGTATCCGG	290	33
	3′ARO	CAAGGGTAAATTCATTGGGCTTGG		
ERα (X61098)	5′ERα	AATTCTGACAATCGACGCCAG	345	29
	3′ERα	GTGCTTCAACATTCTCCCTCCTC		
ERβ (U57439)	5′ERβ	GAAGCTGAACCACCCAATGT	210	36
	3′ERβ	CAGTCCCACCATTAGCACCT		
AR (M23264)	5′AR	TACCAGCTCACCAAGCTCCT	496	26
	3′AR	ACACTGGGCCACAAGAAGAT		
L19 (NM_031103)	5′L19	GAAATCGCCAATGCCAACTC	290	24
	3′L19	ACCTTCAGGTACAGGCTGTG		

Genbank accession numbers are indicated in parentheses.

Steroid determinations

The serum and intratesticular hormonal concentrations were determined by using specific RIA (ICN Pharmaceuticals, Inc, CA, USA). The intra- and inter-assay variation coefficients were 4·6 and 7·5% for testosterone and 6·4 and 5·9% for estradiol. The intratesticular testosterone concentration was performed after homogenization of the testes in 10 ml TEG buffer and was expressed as ng/ml per g of tissue.

AR and ERα immunohistochemistry

Deparaffined sections were hydrated, treated with trypsin solution for 15 min at 37 °C for antigen retrieval, and then treated with 3% hydrogen peroxide solution in methanol for 10 min to block endogenous peroxidase activity. These steps were followed by washing the sections in PBS and subsequently incubated 10 min at room temperature with 10% goat serum to block unspecific binding. The sections were then incubated overnight at 4 °C with specific primary antibody (mentioned above) diluted in PBS with 1% BSA. Sections were then washed in PBS and incubated at room temperature for 30 min with specific secondary antibody (Zymed Laboratories Inc., San Francisco, CA, USA). Sections were washed in PBS, then revealed by treating with liquid diaminobenzidine (Zymed Laboratories), and then counterstained with hematoxylin. The negative controls were processed by replacing the primary antibody with PBS and no indication of staining was observed.

Statistical analysis

All results are means ± S.E.M. Statistical analysis was performed by one-way ANOVA followed by Student–Newman–Keuls test (Sokal & Rohlf 1995). Values of $P<0·05$ were considered significant.

Results

Body and testicular weights

The body weight of pups whose mothers were subjected to a protein-energy (PER) or ER diet during lactation was significantly lower (PER = 58%; ER = 46%) than that of the controls at the end of lactation. The absolute weights of the testes at the end of lactation were significantly decreased in both groups on dietary treatment (65% in the PER and 60% in the ER group). On the other hand, the testicular weight in relation to the body weight showed only a slight reduction in PER and ER groups compared with the C group (Fig. 1).

Hormone concentrations

Compared with the control group, the serum testosterone concentration was significantly increased in the PER group (390%). Serum testosterone concentration in the ER group was slightly, but not significantly, higher than in controls (Fig. 2A). The testicular testosterone concentration was significantly increased in both PER and ER groups compared with controls (210% in the PER and 520% in the ER group, $P<0·01$). The animals of the ER group also showed a significant ($P<0·01$) increase in testicular testosterone concentration when compared with the animals of PER group (Fig. 2B).

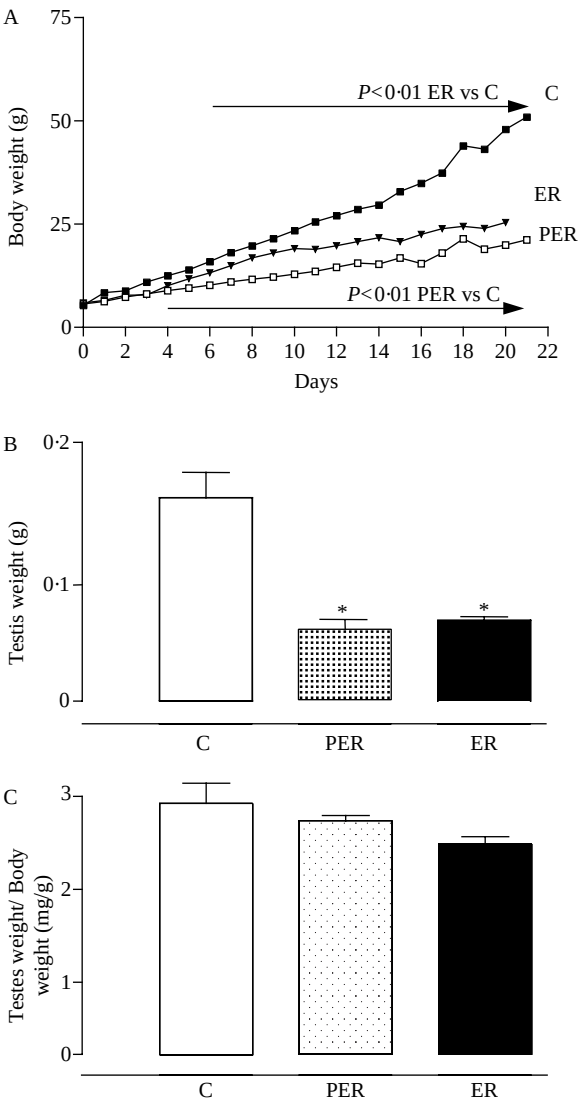

Figure 1 Body (A) and testes (B) absolute weight and the relation of this organ to the body weight (C) of 21-day-old rats whose dams were fed a diet with 23% of the protein – control group (C), a diet with 8% of protein – protein-restricted group (PER), or a diet with 23% of protein but in restricted quantities – energy-restricted group (ER), during the lactation period. Values are given as mean ± S.D. of seven animals per group. *$P<0·001$ vs control.

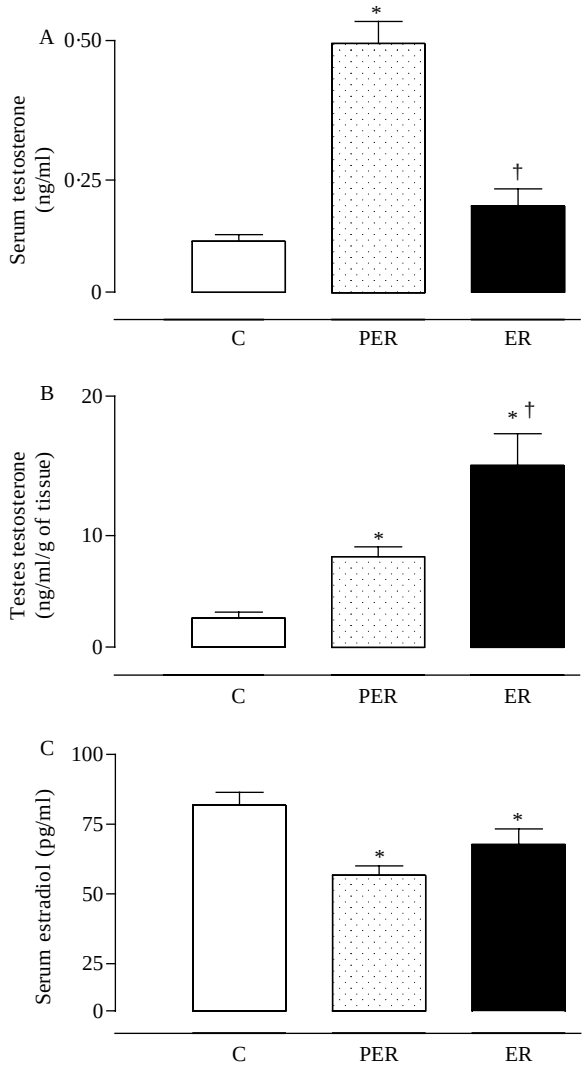

Figure 2 Serum (A) and testicular (B) concentrations of testosterone and serum estradiol concentration (C) of 21-day-old rats whose dams were fed a diet with 23% of protein – control group (C), a diet with 8% of protein – protein-restricted group (PER), or a diet with 23% of protein but in restricted quantities – energy-restricted group (ER), during the lactation period. Values are given as means ± S.D. of seven animals per group. *$P<0{\cdot}01$ vs C; †$P<0{\cdot}01$ vs PER.

The serum estradiol concentrations were significantly decreased in both PER and ER groups compared with controls (PER = 34%, $P<0{\cdot}01$; ER = 19%; Fig. 2C).

Aromatase expression

We performed semi-quantitative RT-PCR in order to determine whether aromatase gene expression in testis is affected by a protein-energy or an ER diet during lactation (Fig. 3). We have observed a significant decrease of more than 70% of the aromatase/L19 ratio in testes of PER and ER groups compared with the control group.

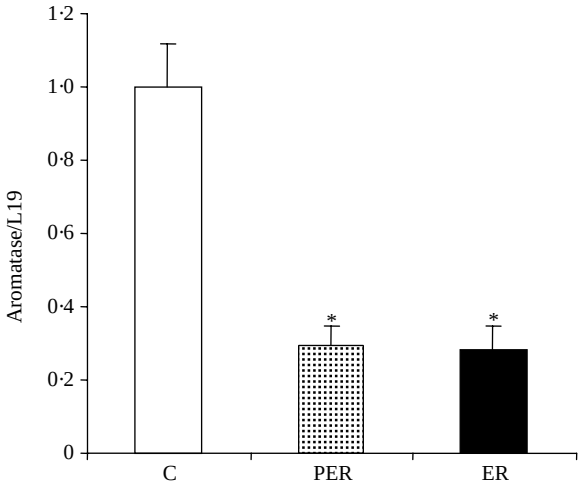

Figure 3 Expression of aromatase gene in testis of protein-energy restricted group (PER) and energy-restricted group (ER). After RT-PCR reactions, the amplified fragments were run on a 2% agarose gel and visualized by u.v. transillumination. The ratios between the signal intensities (arbitrary units) of aromatase and L19 are represented as means ± S.E.M. The aromatase/L19 ratios in testis of PER group ($n=5$) and ER group ($n=4$) are compared with the ratio of control testis ($n=5$) considered to be 1.*$P<0{\cdot}05$ compared with control.

Androgen receptors: mRNA and protein concentrations in testis of protein-energy (PER) and energy-restricted groups (ER)

The concentration of mRNAs recorded as the AR/L19 ratio was significantly increased in the two groups (111% in PER and 120% in ER groups; Fig. 4A). The AR protein expression (Fig. 4B) was also significantly enhanced in both groups (558% in the PER and 120% in the ER groups). The animals of the PER group showed a significant increase (198%) in the androgen testicular receptor protein concentration when compared with the animals of ER group.

Estrogen receptor: mRNA and protein concentrations in testis of protein-energy (PER) and energy-restricted group (ER)

The values of the ERα/L19 ratio are shown in Fig. 5A and it is clear that the amount of transcripts is lower in PER and ER groups compared with control group; however, this diminution was only significant in the PER group. The concentrations of ERβ transcripts were unchanged despite the treatments (data not shown). Conversely, the estrogen receptor-α protein concentration was significantly increased (Fig. 5B) in both PER and ER groups (PER = 617%; ER = 393%).

H&E and Immunohistochemistry of AR and ERα

H&E sections are shown in Fig. 6A (400× magnification) and 6B (1000× magnification). AR immunoreactive staining was strong in most Leydig cells and peritubular myoid cells. Sertoli cells and germ cells (spermatogonia) were also stained (Fig. 7A).

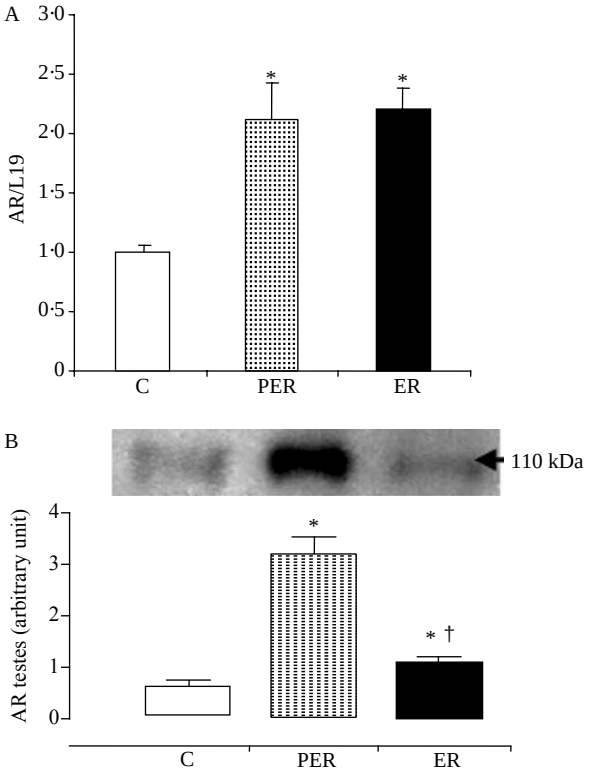

Figure 4 Androgen receptors: transcripts and protein. Expression of *AR* (A) gene in testis of protein-energy restricted group (PER) and energy-restricted group (ER). After RT-PCR reactions, the amplified fragments were run on a 2% agarose gel and visualized by u.v. transillumination. The ratios between the signal intensities (arbitrary units) of AR and L19 are represented as means±S.E.M. The AR/L19 ratios in testes of ER group ($n=5$) and PER group ($n=4$) testes are compared with the ratio of control testis ($n=5$) considered to be 1.*$P<0{\cdot}05$ compared with C. Western blot analysis of testicular androgen receptor protein (B) concentration; 70 μg total protein was added in each lane. Values are given as means±S.D. of seven animals per group. *$P<0{\cdot}05$ vs C; †$P<0{\cdot}001$ vs PER. Molecular weight of the protein is indicated.

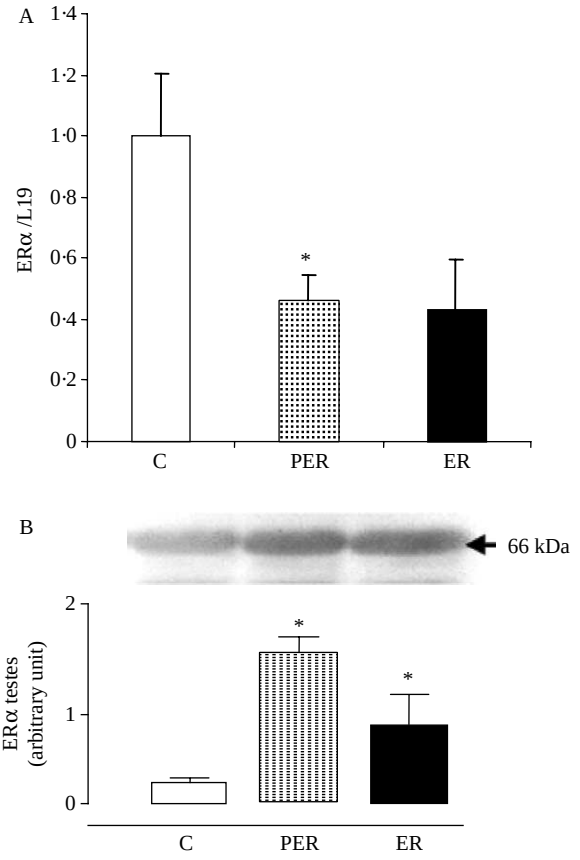

Figure 5 Estrogen receptors: transcripts and protein. Expression of *ERα* gene in testis of protein-energy restricted group (PER) and energy-restricted group (ER). After RT-PCR reactions, the amplified fragments were run on a 2% agarose gel and visualized by u.v. transillumination. The ratios between the signal intensities (arbitrary units) of ERα and L19 (A) are represented as means±S.E.M. The ERα L19 ratios in testes of ER group ($n=4$) and PER group ($n=4$) testes are compared with the ratio of control testes ($n=4$) considered to be 1. Western blot analysis of testicular estrogen receptor α protein (B) concentration of the 21-day-old rats; 70 μg total protein were added in each lane. The molecular weight of the receptor is shown. Values are given as means±S.D. of seven animals per group. *$P<0{\cdot}05$ vs C.

ERα staining was present in Leydig cells, peritubular myoid cells, Sertoli, and germ cells (Fig. 7B). Despite some difference in the intensity of staining, the testes of all the three groups studied showed staining in the same cells.

Discussion

This study provides further evidence that early malnutrition can lead to endocrine disruption (Moura *et al.* 1997, Ramos *et al.* 1997, Rocha de Melo & Guedes 1997, Passos *et al.* 2000, Teixeira *et al.* 2002). The maternal nutritional state during lactation is equivalent and possibly even more important than that during gestation, as evidenced by a study from Léonhard *et al.* (2003) that showed that the offspring whose dams were malnourished during lactation had more drastic consequences on gonadal development when compared with the offspring whose dams were malnourished only during pregnancy or during pregnancy and lactation.

The reduction in body and testes weights observed in this study is in agreement with past results (Nelson *et al.* 1992, Ramos *et al.* 1997, Young *et al.* 2000, Léonhard *et al.* 2003). In spite of the significant reduction in body and testes weights, the difference in the testicular/body weight ratio is not significant in either malnourished groups compared with the control group. The reduction in body and organs weights is independent of the animal's age, nutrient deficit, and duration of the malnutrition (Young *et al.* 2000). Recently, we showed that both PER and ER groups exhibited a significant reduction in the total, luminal, and epithelial areas and in the epithelial height evaluated by stereological methods.

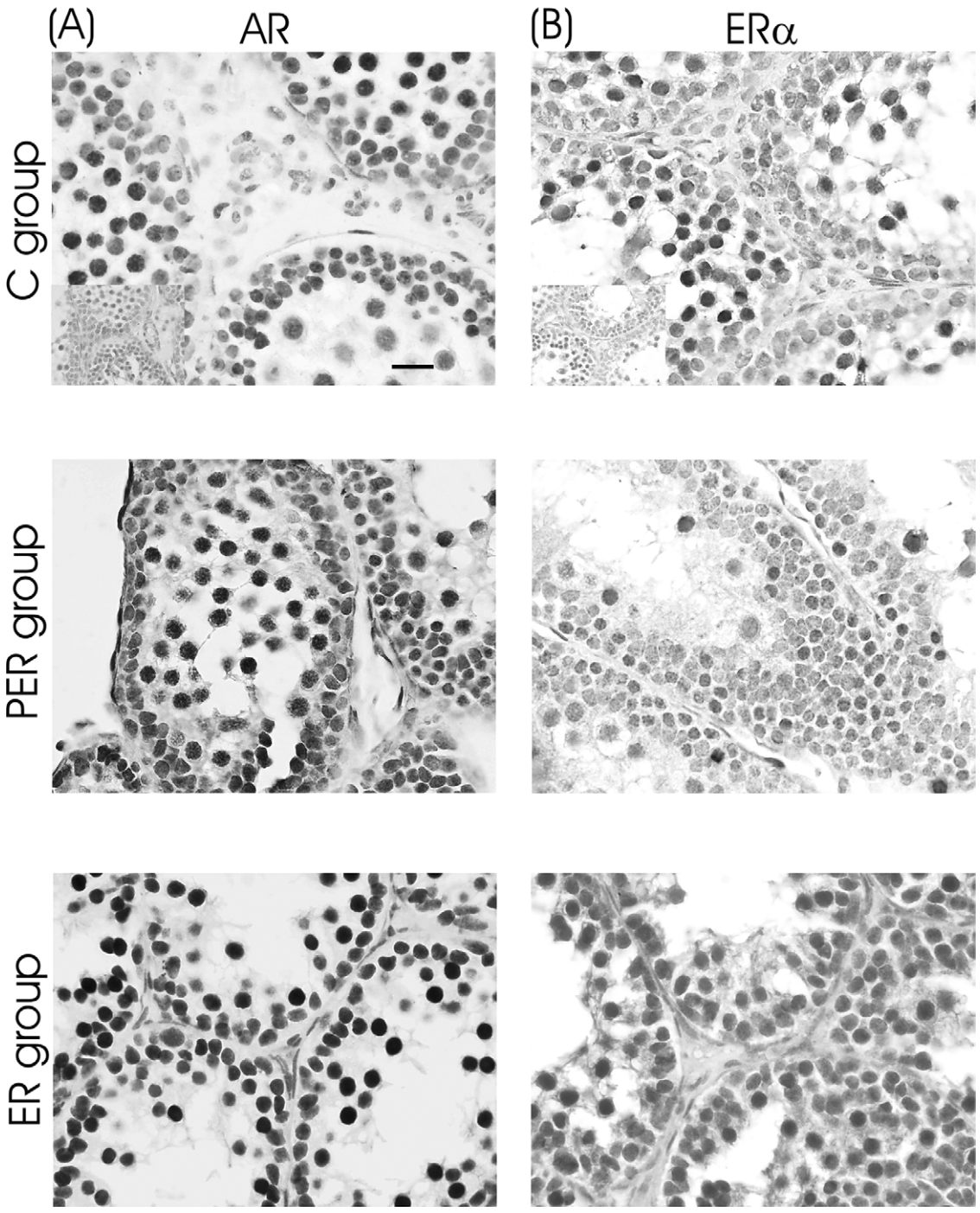

Figure 6 Photomicrographs showing testes of control group, protein-energy restricted group and energy-restricted group stained with H&E. Magnification 400× (A) and 1000× (B). Bar=20 μm.

These structural alterations could contribute to some differences observed in this study and probably have an impact on the capacity of the testes to synthesize and/or metabolize steroids (Ramos *et al*. 2006).

Our past results show that the offspring of mothers that were subjected to protein or energy malnutrition during lactation present an increase in leptin serum concentration (Teixeira *et al*. 2002) that could be related to the low weight

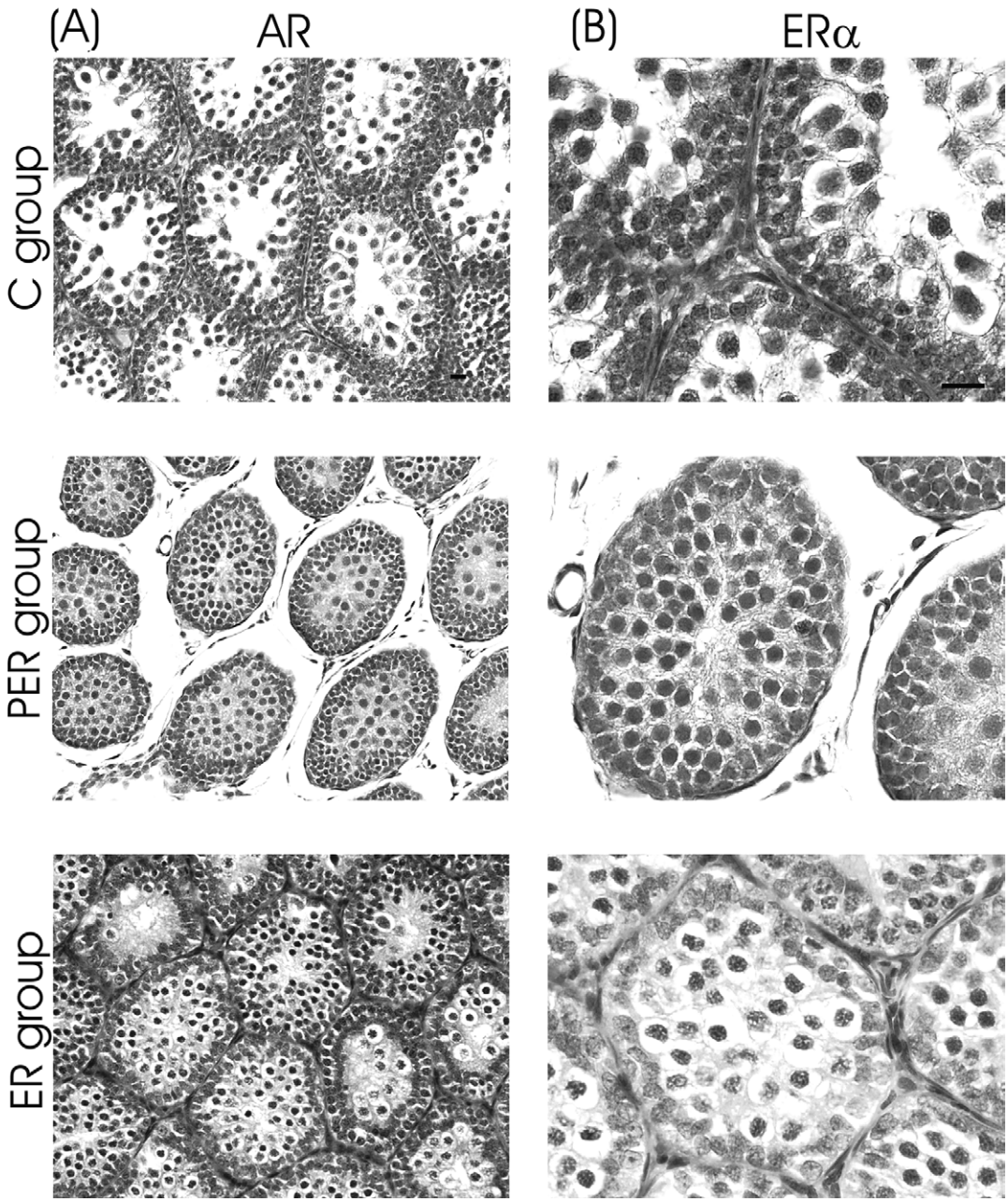

Figure 7 Androgen (AR) and estrogen receptors (ERα) immunohistolocalization. Photomicrographs (A) showing immunohistochemistry for AR in rat testis of control group, protein-energy restricted group, and energy-restricted group. Immunolocalization is detected in Leydig cells, Sertoli cells, peritubular myoid cells, and spermatogonia cells. Magnification 1000×. Photomicrographs (B) showing immunohistochemistry for ERα in rat testis of control group, protein-energy restricted group, and energy-restricted group. Immunolocalization is detected in Leydig cells, Sertoli cells, peritubular myoid cells, and spermatogonia cells. Magnification 1000×. Bar=20 μm. Insert: negative control.

gain of the animals. Conversely, leptin is also a potent stimulator of the LH and FSH production (Caprio *et al.* 2001, Ogura *et al.* 2001). Our results did not agree with others' data showing that food restriction leads to low serum testosterone concentration (Desjardins & Lopez 1983, Nelson *et al.* 1992, Young *et al.* 2000), probably related to differences in the experimental model used, since the age of animals, time, and kind of malnutrition are important factors that could influence the animals in different ways.

In agreement with the literature, we showed in this paper that AR is present in Leydig cells, peritubular myoid cells and Sertoli cells (Sar *et al.* 1990, Bremner *et al.* 1994, Zhou *et al.*

1996, Pelletier *et al*. 2000, Zhu *et al*. 2000). The presence of AR in germ cells is controversial and it was shown only in spermatogonia of prepubertal animals (Zhou *et al*. 1996) what is in agreement with the results observed in this paper.

In relation to ERα, our data are in agreement with the literature showing that ERα immunolocalization is detected in Leydig cells (Saunders *et al*. 1998, Bilinska *et al*. 2001, Zhou *et al*. 2002) and in the peritubular myoid cells (Zhou *et al*. 2002). Sertoli and germ cells were also stained (Pelletier *et al*. 2000). Malnutrition during lactation did not alter the localization of either androgen and estrogen receptors.

In males, testosterone synthesized by Leydig cells is the primary endocrine and/or paracrine factor involved in the regulation of testicular development, initiation of spermatogenesis and formation of secondary sexual characteristics. The testicular testosterone concentration is a determinant factor for spermatogenesis (Wright & Frankel 1979), and the spermatozoa production is related to testosterone concentration (Awoniyi *et al*. 1989, Mc Lachlan *et al*. 1994). The increase in the testicular testosterone concentration observed in both dietary treated groups could be a result of an adaptive mechanism of the Leydig cells as reported elsewhere in other experimental models (Boujrad *et al*. 1995) and/or is the consequence of an abnormal excretion of testosterone.

The increment observed in the serum testosterone concentration in the PER group suggests that this hormone is being normally synthesized and secreted into the circulation. On the other hand, the ER group presented normal concentration of serum testosterone, in spite of an increase in testicular testosterone concentration. In this last group, the synthesis is not correlated with the secretory process, therefore is in favor of an abnormal secretory pathway.

The present results show that independent of the alteration in the synthesis and secretion of testosterone, the androgen receptor (mRNA and protein concentrations) is altered in the same manner as serum testosterone, i.e. a higher increase in the PER group, what could suggest a receptor upregulation. However, it is known that dihydrotestosterone has four times more affinity for the androgen receptor than testosterone (Tapanaien *et al*. 1984); therefore, we cannot discard the possibility of an autoregulation by this hormone, and indeed 5 α reductase activity increases at that age (Tapanaien *et al*. 1984).

The aromatase gene expression is controlled by androgens likely via specific responsive elements leading to either an enhancement (Genissel & Carreau 2001, Lambard *et al*. 2005) or a decrement of the amounts of specific transcripts (Douglas *et al*. 2006). As these animals present low serum estradiol concentration, it is obvious that maternal malnutrition leads to a reduction in the synthesis of aromatase enzyme, which is indeed observed as there is a decrease of more than 65% of the aromatase expression in both groups. This reduction of aromatase expression is correlated with an increase of endogenous testosterone in PER and ER pups. It should also be stressed that the impact of caloric restriction (ER) is more dramatic than that of the reduced protein intake (PER) for most of the parameters determined and especially for blood testosterone and androgen receptor concentrations as also observed in adult rats under the same diet (Marcelly de Souza Santos *et al*. 2004)

Tena-Sempere *et al*. (2000) reported that neonatal exposure to estrogens differentially alters testicular expression of α and β ER mRNAs. ERα mRNA concentrations, as well as those of AR, were significantly decreased, whereas relative and total expression concentrations of ERβ mRNA increased during postnatal/prepubertal development after neonatal estrogen exposure, a phenomenon that was not mimicked by luteinizing hormone-releasing hormone antagonist treatment. The authors suggest that the effect of estrogen on the expression concentrations of AR and ERα mRNAs probably involves a direct action on the developing testis and cannot be attributed to estrogen-induced suppression of gonadotropin secretion during the neonatal period. If estrogens can indeed have a direct action on the developing testis, we can assume that, in our malnutrition experimental model, the increment in the ERα and AR protein concentration expression in both malnourished groups could be a consequence of a lack of the estrogen action on the developing testis since a decrease of the concentration of aromatase transcripts has been observed in both PER and ER groups.

The importance of aromatase which is present in most of the testicular cells and the widespread distribution of ERs in male reproductive tract is obvious (Carreau *et al*. 2003); thus, any changes induced by diet which will reduce the amount of estrogens will likely affect testicular development, and thus spermatogenesis as shown elsewhere (Bilinska *et al*. 2004).

We can surmise that an adequate nutritional state in early life is important for normal maturation of the male gonad and steroidogenic pathway on the one hand, and in the expression of steroid receptors on the other hand. Gene expression and protein synthesis are therefore under several hormonal controls which depend on numerous factors including protein and energy as demonstrated in the diet studies herein. The reduced protein intake is less deleterious than the caloric restriction, but both affect the testes of male pups, which could lead to abnormal testicular development and may delay the onset of spermatogenesis for which a correct androgens/estrogens ratio is absolutely required.

Acknowledgements

This work was supported by grants from the National Council of Scientific and Technological Development (CNPq), Foundation for Research Support of Rio de Janeiro (FAPERJ), Coordination for Improvement of Post-Graduated Students (CAPES), and State University of Rio de Janeiro (UERJ–SR2), Brazil. Additional support was provided by a grant from the French National Institute of Research in Agronomy. The authors declare that there is no

conflict of interest that would prejudice the impartiality of this scientific work.

References

Awoniyi CA, Santulli R, Sprando RL, Ewing LL & Zirkin BR 1989 Restoration of advanced spermatogenic cells in the experimentally regressed rat testis: quantitative relationship to testosterone concentrations within the testis. *Endocrinology* **124** 1217–1223.

Bayne K 1996 Revised guide for the care and use of laboratory animals available. American physiological society. *Physiologist* **39** 208–211.

Bilinska B, Shamalz-Fraczek B, Kotula M & Carreau S 2001 Photoperiod dependent capability of androgen aromatization and the role of estrogens in bank vole testis visualized by means of immunohistochemistry. *Molecular and Cellular Endocrinology* **178** 189–198.

Bilinska B, Gancarczyk M, Paziewska A & Carreau S 2004 Dose and photoperiod dependent effcet of 17β-estradiol or ICI 182,780 administration on testicular structure, acceleration of spermatogenesis and aromatase immunoexpression in immature bank voles. *Acta Histochemica* **106** 269–278.

Boujrad N, Hochereau de reviers MT & Carreau S 1995 Evidence for a germ cell control of Sertoli cell function in three models of germ cell depletion in adult rat. *Biology of Reproduction* **53** 1345–1352.

Bradford MM 1976 A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72** 248–254.

Bremner WJ, Millar MR, Sharpe RM & Saunders PT 1994 Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. *Endocrinology* **135** 1227–1234.

Caprio M, Fabbrini E, Isidori AM, Aversa A & Fabbri A 2001 Leptin in reproduction. *Endocrinology and Metabolism* **12** 65–72.

Cardone A, Angelini F & Varriale B 1998 Autoregulation of estrogen and androgen receptor mRNAs and down regulation of androgen receptor mRNA by estrogen in primary cultures of lizard testis cells. *General and Comparative Endocrinology* **110** 227–236.

Carreau S, Genissel C, Bilinska B & Levallet J 1999 Topical review. Sources of estrogens in the testis and the reproductive tract of the male. *International Journal of Andrology* **22** 211–223.

Carreau S, Lambard S, Delalande C, Galeraud-Denis I, Bilinska B & Bourguiba S 2003 Aromatase expression and role of estrogens in male gonad: a review. *Reproductive Biology and Endocrinology* **1** 35–40.

Chen H, Luo L, Liu J, Brown T & Zikin BR 2005 Aging and caloric restriction: effects on Leydig cell steroidogenesis. *Experimental Gerontology* **40** 498–505.

Chik CL, Ho AK & Brown GM 1987 Effect of food restriction on 24-h serum and pineal melatonin content in male rats. *Acta Endocrinologica* **115** 507–513.

Chik CL, Ho AK & Brown GM 1989 Effect of pinealectomy on the undernutrition-induced suppression of the reproductive axis in rats. *Acta Endocrinologica* **120** 569–573.

Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162** 156–159.

Cónsole GM, Jurado SB, Oyhenart E, Ferese C, Pucciarelli H & Gómez-Dumm CLA 2001 Morphometric and ultrastructural analysis of different pituitary cell populations in undernourished monkeys. *Brazilian Journal of Medical and Biological Research* **34** 65–74.

Dai JL, Maiorino CA, Gkonos PJ & Burnstein KL 1996 Androgenic up-regulation of androgen receptor cDNA expression in androgen-independent prostate cancer cells. *Steroids* **61** 531–539.

Desjardins C & Lopez MJ 1983 Environmental cues evoke differential responses in pituitary–testicular function in deer mice. *Endocrinology* **112** 1398–1406.

Dong Q, Bergendahl M, Huhtaniemi I & Handelsmen DJ 1994 Effect of undernutrition on pulsatile luteinizing hormone (LH) secretion in castrate and intact male rats using an ultrasensitive immunofluorometric LH assay. *Endocrinology* **135** 745–750.

Douglas ML, Richardson MM & Nicol DL 2006 Testicular germ cell tumors exhibit evidence of hormone dependence. *International Journal of Cancer* **118** 98–102.

Fishbeck KL & Rasmussen KM 1987 Effect of repeated cycles on maternal nutritional status, lactational performance and litter growth in *ad libitum*-fed and chronically food-restricted rats. *Journal of Nutrition* **117** 1967–1975.

Genissel C & Carreau S 2001 Regulation of the aromatase gene expression in mature rat Leydig cells. *Molecular and Cellular Endocrinology* **178** 141–148.

Gonzalez-Cadavid NF, Vernet D, Navarro AF, Rodriguez AJ, Swerdloff RS & Rajfer J 1993 Up-regulation of the concentrations of androgen receptor and its mRNA by androgens in smooth-muscle cells from rat penis. *Molecular and Cellular Endocrinology* **90** 219–229.

Grewal T, Mickeelsen D & Hafs HD 1971 Androgen secretion and spermatogenesis in rat following semistarvation. *Proceedings of the Society for Experimental Biology and Medicine* **138** 723–729.

Hackenberg R, Hawighorst T, Filmer A, Slater EP, Bock K, Beato M & Schulz KD 1992 Regulation of androgen receptor mRNA and protein concentration by steroid hormones in human mammary cancer cells. *Journal of Steroid Biochemistry and Molecular Biology* **43** 599–607.

Herbert DC 1980 Morphology of the mammotrophs and gonadotrophs in the anterior pituitary gland of rats with protein-calorie malnutrition. *American Journal of Anatomy* **158** 521–531.

Hess RA 2000 Oestrogen in fluid transport in efferent ducts of the male reproductive tract. *Reviews of Reproduction* **5** 84–92.

Howland BE 1975 The influence of feed restriction and subsequent refeeding on gonadotrophin secretion and serum testosterone concentrations in male rats. *Journal of Reproduction and Fertility* **44** 429–436.

Kerr JE, Allore RJ, Beck SG & Handa RJ 1995 Distribution and hormonal regulation of androgen receptor (AR) and AR messenger ribonucleic acid in the rat hippocampus. *Endocrinology* **136** 3213–3221.

Krongrad A, Wilson CM, Wilson JD, Allman DR & McPhaul MJ 1991 Androgen increases androgen receptor protein while decreasing receptor mRNA in LNCaP cells. *Molecular and Cellular Endocrinology* **76** 79–88.

Lambard S, Silandre D, Delalande C, Galeraud-Denis I, Bourguiba S & Carreau S 2005 Aromatase in testis: expression and role in male reproduction. *Journal of Steroid Biochemistry and Molecular Biology* **95** 63–69.

Léonhard M, Lesage J, Croix D, Dutriez-Casteloot I, Beauvillain JC & Dupouy JP 2003 Effects of perinatal maternal food restriction on pituitary–gonadal axis and plasma leptin concentration in rat pup at birth and weaning and on timing of puberty. *Biology of Reproduction* **68** 390–400.

Lindzey J, Kumar MV, Grossman M, Young C & Tindall DJ 1994 Molecular mechanisms of androgen action. *Vitamins and Hormones* **49** 383–432.

Marcelly de Souza Santos A, Ferraz MR, Teixeira CV, Sampaio FJB & da Fonte Ramos C 2004 Effects of undernutrition on serum and testicular testosterone concentrations and sexual function in adult rats. *Hormone and Metabolic Research* **36** 27–33.

Mc Lachlan RI, Wreford NG, Tsonis CKDM & Robertson DM 1994 Testosterone effects on spermatogenesis in the gonadotrophin-releasing hormone-immunized rat. *Biology of Reproduction* **50** 271–280.

Moura AS, Caldeira-Filho JS, Mathias PC & Franco de Sá CCN 1997 Insulin secretion impairment and insulin sensitivity improvement in adult rats undernourished during early lactation. *Research Communications in Molecular Pathology and Pharmacology* **96** 180–192.

Nastiuk KL & Clayton DF 1994 Seasonal and tissue-specific regulation of canary androgen receptor messenger ribonucleic acid. *Endocrinology* **134** 640–649.

Nelson RJ, Kita M, Blom JMC & Rhyne-Grey J 1992 Photoperiod influences the critical caloric intake necessary to maintain reproduction among male deer mice (*Peromyscus maniculatus*). *Biology of Reproduction* **46** 226–232.

Ogura K, Irahara M, Kiyokawa M, Tezuda M, Matsuzaki T, Yasui T, Kamada M & Aono T 2001 Effects of leptin on secretion of LH and FSH from primary cultured female rat pituitary cells. *European Journal of Endocrinology* **144** 653–658.

Passos MCF, Ramos CF & Moura EG 2000 Short and long term effects of malnutrition in rats during lactation on the body weight of offspring. *Nutrition Research* **20** 1603–1612.

Pelletier G, Labrie C & Labrie F 2000 Localization of oestrogen receptor alpha, oestrogen receptor beta and androgen receptors in the rat reproductive organs. *Journal of Endocrinology* **165** 359–370.

Quarmby VE, Yarbrough WG, Lubahn DB, French FS & Wilson EM 1990 Autologous down-regulation of androgen receptor messenger ribonucleic acid. *Molecular Endocrinology* **4** 22–28.

Ramos CF, Lim APS, Teixeira CV, Brito PD & Moura EG 1997 Thyroid function in post-weaning rats, which dams were fed a low protein diet during suckling. *Brazilian Journal of Medical and Biological Research* **30** 133–137.

Ramos CF, Teixeira CV, Passos MCF, Pazos-Moura CC, Lisboa PC, Curty FH & Moura EG 2000 Low-protein diet changes thyroid function in lactating rats. *Proceedings of the Society for Experimental Biology and Medicine* **224** 256–263.

Ramos CF, Silva AM, Costa WS & Sampaio FJB 2006 Stereological evaluation of the seminiferous tubules of rats after maternal undernutrition during the lactation period. *Urologia Internationalis* **76** 63–66.

Reeves PG, Nielsen FH & Fahey GC 1993 AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition *Ad Hoc* Writing Committee on the reformulation of the AIN-76 rodent diet. *Journal of Nutrition* **123** 1939–1951.

Rocha de Melo AP & Guedes RCA 1997 Spreading depression is facilitated in adult rats previously submitted to short episodes of malnutrition during the lactation period. *Brazilian Journal of Medical and Biological Research* **30** 663–669.

Sar M, Lubahn DB, French FS & Wilson EM 1990 Immunohistochemical localization of the androgen receptor in rat and human tissues. *Endocrinology* **127** 3180–3186.

Saunders PTK, Fisher JS, Sharpe RM & Millar MR 1998 Expression of oestrogen receptor beta (ERβ) occurs in multiple cell types, including some germ cells, in the rat testis. *Journal of Endocrinology* **156** R13–R17.

Sharpe RM 1998 The roles of oestrogen in the male. *Trends in Endocrinology and Metabolism* **9** 371–378.

Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, It Y, Fisher CR, Dodson MM *et al.* 1994 Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrine Reviews* **15** 342–355.

Simpson ER, Rubin G, Clyne CD, Robertson KM, O'Donnell L, Jones ME & Davis S 2000 The role of local estrogen biosynthesis in males and females. *Trends in Endocrinology and Metabolism* **11** 184–188.

Tapanaien J, Kuopio T, Pelliniemi LJ & Huhtaniemi I 1984 Rat testicular endogenous steroids and number of Leydig cells between the fetal period and sexual maturity. *Biology of Reproduction* **31** 1027–1035.

Teixeira CV, Passos MCF, Ribeiro CRS, Dutra SP & Moura EG 2002 Leptin serum concentration, food intake and body weigh in rats whose mothers were exposed to malnutrition during lactation. *Journal of Nutritional Biochemistry* **13** 493–498.

Tena-Sempere M, Navarro J, Pinilla L, González LC, Huhtaniemi I & Aguilar E 2000 Neonatal exposure to estrogen differentially alters estrogen receptor α and β mRNA expression in rat testis during postnatal development. *Journal of Endocrinology* **165** 345–357.

Tena-Sempere M, Barreiro ML, Gonzalez LC, Gaytan F, Zhang FP, Caminos JE, Pinilla L, Casanueva FF, Dieguez C & Aguilar E 2002 Novel expression and functional role of ghrelin in rat testis. *Endocrinology* **143** 717–725.

Turner KJ, Morley M, MacPherson S, Millar MR, Wilson JA, Sharpe RM & Saunders PTK 2001 Modulation of gene expression by androgen and oestrogen in the testis and prostate of the adult rat following androgen withdrawal. *Molecular and Cellular Endocrinology* **178** 73–87.

Wolf DA, Herzinger T, Hermeking H, Blaschke D & Hürz W 1993 Transcriptional and posttranscriptional regulation of human androgen receptor expression by androgen. *Molecular Endocrinology* **7** 924–936.

Wright WW & Frankel AI 1979 Endogenous androgen concentrations in nuclei isolated from seminiferous tubules of mature rat testis. *Journal of Steroid Biochemistry* **10** 633–640.

Young KA, Zirkin BR & Nelson RJ 2000 Testicular regression in response to food restriction and short photoperiod in White-Footed Mice (*Peromyscus leucopus*) is mediated by apoptosis. *Biology of Reproduction* **62** 347–354.

Zhou ZX, Wong CI, Sar M & Wilson EM 1994 The androgen receptor. *Recent Progress in Hormone Research* **49** 249–274.

Zhou X, Kudo A, Kawakami H & Hirano H 1996 Immunohistochemical localization of androgen receptor in mouse testicular germ cells during fetal and postnatal development. *Anatomical Record* **245** 509–518.

Zhou Q, Nie R, Prins GS, Saunders PTK, Katzenellenbogen BS & Hess RA 2002 Localization of androgen and estrogen receptors in adult male mouse reproductive tract. *Journal of Andrology* **23** 870–881.

Zhu LJ, Hardy MP, Inigo IV, Huhtaniemi I, Bardin CW & Moo-Young AJ 2000 Effects of androgen on androgen receptor expression in rat testicular and epididymal cells: a quantitative immunohistochemical study. *Biology of Reproduction* **63** 368–376.

Received in final form 30 November 2006
Accepted 1 December 2006
Made available online as an Accepted Preprint
11 December 2006