Evaluation of Ki-67 antigen expression in the zona reticularis of the adrenal cortex of female rats in persistent estrus

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BACKGROUND: Hyperandrogenism and chronic anovulation are basic characteristics of polycystic ovary syndrome (PCOS), and androgens from the adrenal glands play an important role in the hyperandrogenism. Our aim was to evaluate the proliferative activity in the zona reticularis cells of the adrenal cortex of female rats in persistent estrus, a model developed to mimic PCOS.

METHODS: Forty-four female Wistar–Hannover rats were randomly divided into two groups: control (n = 17) and animals which received 1.25 mg testosterone propionate s.c. on the second day of life (n = 27). At 90 days of age, after confirmation of persistent estrus, the animals were sacrificed, and the adrenal glands were removed and fixed in 10% buffered formalin to investigate Ki-67 antigen (marker of proliferation) expression by immunohistochemical analysis. Student’s t-test and Levene’s test were used in the statistical analysis.

RESULTS: The mean percentage of Ki-67-stained nuclei per 1000 cells in the zona reticularis of the adrenal cortex was 15.58 ± 1.14 (SEM) and 51.59 ± 1.81 in the control and persistent estrus animals, respectively (P < 0.001).

CONCLUSIONS: Proliferative activity in the zona reticularis cells of the adrenal cortex of the androgenized female rats was significantly greater than that of the control animals.

Key words: persistent estrus / rat / polycystic ovary / adrenal / Ki-67

Introduction

Polycystic ovary syndrome (PCOS) is a heterogenous, reproductive and metabolic disorder that affects 5–10% of women of reproductive age, thereby constituting the most common human female endocrinopathy (Zhou et al., 2005; Xita and Tsatsoulis, 2006). The etiology of PCOS is unclear and it is characterized by chronic anovulation and hyperandrogenism (Singh, 2005; Yildiz et al., 2006; Baillargeon and Carpentier et al., 2007). Although the ovaries are the principal source of androgen production in PCOS patients, elevated androgen levels, particularly dehydroepiandrosterone sulphate (DHEAS), have also been found in the adrenal gland of around 40–70% of patients (Hoffman et al., 1984; Carmina et al., 1992; Aziz et al., 1998). Although DHEAS levels decrease with age, another study has nevertheless found that around 25% of women with PCOS may be considered to have elevated androgen levels from the adrenals (Moran et al., 2004).

The cortical region of the adrenal gland of mammals is composed of three morphologically and functionally distinct zones, the zona glomerulosa, the zona fasciculata and the zona reticularis, each of which secrete specific corticosteroids (Mitani et al., 2003; da Silva et al., 2007). Inherited abnormalities in steroid biosynthesis resulting in an inability to synthesize the corticosteroids aldosterone and cortisol lead to adrenal cortex hyperplasia and increased androgen production in the zona reticularis (White et al., 1994). Nevertheless, despite the importance of the participation of the adrenal gland in PCOS, for ethical reasons it is impossible to investigate the proliferative activity of the androgen-producing zona reticularis directly in humans. Consequently, evaluating animal models that mimic PCOS may be useful despite the known limitations with respect to extrapolating the results to human beings.
Therefore, since hyperandrogenism and chronic anovulation are basic characteristics of PCOS, for the purposes of the present study a biological model of androgenized rats, or rats in persistent estrus, which has already been tested in other studies, was developed to mimic PCOS (Barraclough, 1961; Gorski and Wagner, 1965; Harris and Levine, 1965; Wrenn et al., 1969; da Silva et al., 2002; da Silva et al., 2007). Although the state of persistent estrus may be achieved by various techniques, the most commonly used method is the s.c. injection of testosterone propionate in newborn rats (Barraclough, 1961), which induces chronic anovulatory syndrome characterized by permanent keratinization of the vaginal epithelium (Barraclough, 1961; Gorski and Wagner, 1965; Harris and Levine, 1965; Wrenn et al., 1969; da Silva et al., 2002; da Silva et al., 2007).

A literature search revealed only one study in which the thickness of the zones of the adrenal cortex of androgenized rats was evaluated morphometrically, and the zona reticularis of these animals was found to be significantly thicker compared with that of control animals (da Silva et al., 2007). Although 17-hydroxylase (CYP17), responsible for the production of cortisol and androgens in the adrenal cortex, has been generally considered to be absent from the adrenal cortex of rats, Pignatelli et al. (2006) showed a relative increase in zona reticularis, and peaks of circulating cortisol, androstenedione and 17-OH-progesterone around post-natal days 16–20 (clearly before the development of gonads, which begins at 30–35 days) suggesting that the rat adrenal has the capacity to secrete steroids arising from 17-hydroxylase, and at this time may contribute to a process similar to human adrenarche. Probably, the mitotic activity of the zona reticularis of androgenized rats is different from that of normal rats. To the best of our knowledge, no reports are available on the proliferative activity of the zona reticularis cells of the adrenal cortex in this model of rat mimicking PCOS, which led to the conception of the present study.

Materials and Methods

Animals

In this study, 44 female virgin Wistar-Hannover rats were used, following the general guidelines established by the Brazilian College for Animal Experimentation (COBEA). The animals were randomly divided into two groups: Group A (control, n = 17) and Group B (study, n = 27). The animals in Group A received 0.1 ml of corn oil (vehicle) subcutaneously on the second day of life, while androgenization or persistent estrus was obtained in the animals of Group B by the application of a s.c. injection of 1.25 mg of testosterone propionate diluted in 0.1 ml of corn oil, also on the second day of life. At 90 days of age, the state of persistent estrus was confirmed in the animals based on the presence of obliteration of the distal third of the vagina (Wrenn et al., 1969), keratinization of the vaginal epithelium, the principal characteristic of persistent estrus, and also on the presence of polycystic ovaries, as observed during histology performed at the time of autopsy. The animals were sacrificed and their adrenal glands were removed, then fixed in 10% buffered formalin for 24 h for immunohistochemical evaluation of the zona reticularis of the adrenal cortex. Only one adrenal gland from each animal was used for the study.

Ki-67 immunohistochemistry

The adrenal glands were cut into 3-μm-thick sections. Next, they were processed and stained with hematoxylin-eosin for morphological evaluation, following which the sections were deparaffinized in xylol for 5 min, dehydrated in absolute ethanol and washed in buffered saline solution at pH 7.4 for 5 min. Immunohistochemical evaluation of the Ki-67 marker was performed using the Envision™ (Dako, USA) detection system, in combination with an antigen recovery method. For this, the sections were treated with 3% hydrogen peroxide diluted in buffered solution for 5 min to block the endogenous peroxide. After recovery of the epitopes, the tissue samples were incubated with primary mouse anti-Ki-67 monoclonal antibody (clone MIB-5/Dako/1:100) for 16 h, that included an overnight period in a refrigerator, at approximately 4°C. Following washing with buffered saline solution, the sections were incubated for 45 min with the Novo Link (Polymer) detection system. To read the reaction, all the sections were treated with a solution of 3,3-diaminobenzidine tetrahydrochloride at a concentration of 1 mg/ml of Tris buffered solution and hydrogen peroxide solution for 5 min. Next, the sections were counterstained with Harris hematoxylin or methyl green for 5 min followed by dehydration in ethyl alcohol and xylol baths. The cells were considered positive for the immunohistochemical expression of the Ki-67 antigen when the nucleus was stained a brownish color.

Quantification

Quantification was carried out by two independent observers who were blinded with respect to which group the rats belonged. Assessment was performed using a Nikon E400 light microscope connected to a Samsung color digital video-camera, model SCC-131, which captured the image and transmitted it to a computer equipped with the Imagelab® software program, version 2.3, developed by Sofium Informática Ltda (São Paulo, Brazil) for image analysis. For evaluation of Ki-67 expression, 1000 cells of the zona reticularis of the adrenal cortex were counted in each slide, whether they were stained by the anti-Ki-67 MIB-5 antibody or not, using a magnification of ×400 and beginning with the area of greatest Ki-67 expression close to the adenral medulla. In each case, the percentage of stained cells was obtained from the ratio of the number of cells with stained nuclei to the total number of cells, multiplied by 100.

Statistical analyses

Analysis of variance and the Tukey–Kramer multiple comparison test were used to analyze the weight of the animals. Comparison of the mean proportions of cells with stained nuclei in the two groups was performed using Student’s t-test for paired samples following Levene’s test for equality of variances. In order to evaluate the agreement of the Ki-67 measures of the two observers, the Pearson correlation coefficient was calculated (Bland and Altman, 1986). A P-value <0.05 was considered statistically significant.

Results

The mean weight of the animals in groups A and B at 90 days was 202 and 250 g, respectively, and this difference was statistically significant (P < 0.0001). The adrenal glands of the rats in persistent estrus were found to be macroscopically larger and darker in color compared with the control group. There was a greater concentration of Ki-67-stained nuclei in the zona reticularis of the adrenal glands of the rats in persistent estrus compared with the animals in the control group (Figs 1 and 2). Quantitative analysis showed mean percentages of Ki-67-stained nuclei per 1000 cells of 15.58 ± 1.14 and 51.59 ± 1.81 in the rats in the control and persistent estrus groups,
respectively, \( P < 0.001 \) (Table I). The box plot in Fig. 3 clearly shows the difference between the mean percentage of Ki-67-stained nuclei in the control and study groups. The correlation coefficients between the measures of the two observers are 0.9350 and 0.9731 for the control and androgenized rats, respectively. The difference of measures of observer 1 and 2, according with Bland-Altman analysis, was not significant.

### Discussion

Polycystic ovaries, chronic anovulation and hyperandrogenism are three key characteristics that have been proposed for the diagnosis of PCOS (Norman et al., 2007). In 2003, at the Rotterdam American Society for Reproductive Medicine/European Society for Human Reproduction and Embryology (ESHRE/ASRM) Consensus workshop, an attempt was made to standardize the working definition of PCOS. Since then, the presence of two of three of the following criteria have been required for the diagnosis of PCOS: (i) oligo and/or anovulation, (ii) clinical and/or biochemical signs of hyperandrogenism and (iii) echographic polycystic ovaries, after the exclusion of other pathologies with a similar clinical presentation, such as congenital adrenal hyperplasia, Cushing’s syndrome and androgen-secreting tumors (The Rotterdam ESHRE/ASRM—Sponsered PCOS Consensus Workshop Group, 2004; Belosi et al., 2006).

Hyperandrogenism is the most constant and prominent diagnostic component of PCOS, which is assessed in accordance with clinical characteristics, biochemical indexes or both (Norman et al., 2007). Unfortunately, serum analysis fails to measure biochemical hyperandrogenism in around 20–40% of patients with PCOS (Chang et al., 2005). The majority of total serum testosterone assays are not designed or validated for detection of values within the normal range for women (Vermeulen et al., 1999), raising concern with respect to their real diagnostic value. The androgens from the

### Table I

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>SEM</th>
<th>Maximum</th>
<th>Minimum</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>17</td>
<td>15.58</td>
<td>1.14</td>
<td>21.50</td>
<td>5.60</td>
</tr>
<tr>
<td>B</td>
<td>27</td>
<td>51.59*</td>
<td>1.81</td>
<td>75.60</td>
<td>34.60</td>
</tr>
</tbody>
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*The difference was statistically significant \( (P < 0.001) \).
adrenal glands, particularly DHEAS, play an important role in hyperandro
genism in patients with PCOS (Hoffman et al., 1984; Carmina et al.,
1992; Azziz et al., 1998); however, for the above-mentioned reasons
their measurement is of little value in the average clinical setting
(Norman et al., 2007). Nonetheless, in view of these reasons, bio-
chemical evaluation of the function of the androgen-producing zone
of the adrenal gland has limited value.

In the present study, in spite of the controversies around steroid
production in normal rats arising from 17-hydroxylation, we used rats
in persistent estrus to mimic PCOS, and the proliferative activity
of the zona reticularis cells of the adrenal glands of these androgenized
rats was found to be significantly greater than that of the control
animals. The development of an ideal animal model that would
reflect the heterogeneity found in human PCOS has been difficult
(Singh, 2005). Over recent decades, investigators have used other
animal models such as mice, hamsters, guinea pigs, and subhuman pri-
mates to study the reproductive cycle and hormonal changes associ-
ated with the physiology of the chronic anovulatory syndrome
(Singh, 2005). However, laboratory rats offer various advantages for
the investigation of PCOS compared with other animal models in
view of their relatively small size, high reproductive rate and the avail-
ability of numerous different genetic strains (Singh, 2005). Barraclough
(1961) was the first to identify the period of highest hypothalamic sen-
sitivity in rats by inducing persistent estrus in all the animals by means
of a simple s.c. injection of 1.25 mg of testosterone propionate during
the first 5 days of life. In adulthood, all these animals developed
characteristics that mimicked PCOS, including chronic anovulation,
sterility, polycystic ovaries, excess weight and aggression when in
contact with males (Barraclough, 1961; Gorski and Wagner, 1965;
Harris and Levine, 1965; Wrenn et al., 1969; da Silva et al., 2002;
da Silva et al., 2007).

The increase in the proliferative activity of the zona reticularis cells
of the adrenal cortex of the androgenized rats used in this study is in
agreement with a previous morphometric study that showed an increase
in the thickness of the zona reticularis of the adrenal glands of androgenized
rats compared with those of control animals (da Silva et al., 2007).
Nevertheless, PCOS is a multifactorial syndrome, and probably no single animal model is able to reproduce all the
aspects of the syndrome. Therefore, it is not clear whether the
results of the present study could differ from other animal models
of PCOS, such as exposure to estradiol-valeter, dehydroepiandroste-
erone or constant-light. The androgenized rats in this study were sig-
ificantly heavier than the controls; however, we did not measure the
amount of fat tissue in the two groups. On the other hand, the greater
body weight of androgenized rats would not explain our findings, since
a prior study showed that the greater thickness of the adrenal cortex
in these animals was due to the greater thickness principally of the
zona reticularis, but no difference was found between the two
groups with respect to the thickness of the zona fasciculata (da Silva
et al., 2007).

In the present study, apoptosis was not measured; only proliferative
activity of the zona reticularis of the adrenal cortex of the animals in
which immunohistochemistry was performed using an anti-Ki-67
(MIBS) antibody, in view of the sensitivity of this technique (Gerdes,
1990; da Silva et al., 2006). Ki-67 is a nuclear antigen present in all pro-
liferating cells in the active phases of the cell cycle, i.e. G1, S, G2 and
mitosis, but which is absent in G0 cells (Gerdes, 1990), unlike other
much used proliferation markers, particularly proliferating cell nuclear antigen (PCNA) which clearly identifies non-proliferating
cells, possibly reflecting the fact that the PCNA gene product is
present in all the cells throughout the cell cycle (Holt et al., 1997).

The etiology of excessive androgen production in the adrenal
glands of women with PCOS is unknown. On the other hand,
there are controversies on the production of steroids by the
adrenal cortex of normal rats. According to some authors, the con-
tribution of the zona reticularis cells to the basal output of any
steroid by the cells of the inner two zones of the adrenal cortex of
rat is relatively small and instead of cortisol, corticosterone is the
main glucocorticoid produced by the adrenal gland in this species
(Bell et al., 1979). This could be explained by the lack of CYP17 (Pel-
letier et al., 2001). On the other hand, Pignatelli et al. (2006) have
reported the production of steroids arising from 17-hydroxylation
in prepubertal rats and this could indicate the capacity of the rat
adrenal to use CYP17 to produce steroids. Nevertheless, it is not
known whether the greater proliferative activity of zona reticularis
cells of the androgenized female rats observed in this study is associ-
ated with CYP17 activity in these persistent estrus rats. More studies
need to be conducted to evaluate not only the activity of CYP17 but
also serum DHEAS levels and other parameters including evaluation
of glucose intolerance and serum lipids, that are part of the metabolic
spectrum of PCOS in order to obtain more data on the profile of this
animal model.

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Submitted on September 10, 2008; resubmitted on November 14, 2008; accepted on November 19, 2008.