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Hormone levels are associated with clinical markers and cytokine levels in human localized cutaneous leishmaniasis

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ABSTRACT

Leishmaniasis is a serious health problem in several parts of the world, and localized cutaneous leishmaniasis (LCL) is the most frequent presentation of the tegumentary form of this disease cluster. Clinical presentations of leishmaniasis are influenced by both parasite and host factors, with emphasis on the host immune response. Alterations in plasma hormone levels have been described in many infections, and changes in hormone levels could be related to an imbalanced cytokine profile. In the present work, we evaluated a group of patients with LCL to determine changes in plasma hormone levels (cortisol, DHEA-S, estradiol, prolactin and testosterone) and their association with clinical markers of disease (lesion size, dose used to reach cure and time to cure) and with cytokines produced by PBMC stimulated by SLA (IFN-γ, IL-10 and TNF-α). Individuals with LCL exhibited lower plasma levels of DHEA-S, prolactin and testosterone compared with sex-matched controls, whereas levels of cortisol and estradiol were similar between patients and controls. Plasma levels of cortisol, estradiol or prolactin positively correlated with at least one clinical parameter. Cortisol and prolactin levels exhibited a negative correlation with levels of IFN-γ, whereas no correlation was observed with IL-10 or TNF-α levels. A decrease in DHEA-S levels was observed in male LCL patients when compared to male healthy controls. No other differences between the sexes were observed. Our results indicate a role for neuroendocrine regulation that restricts Th1 responses in human LCL. It is possible that, although impairing parasite killing, such neuroimmunomodulation may contribute to limiting tissue damage.

1. Introduction

Leishmaniasis comprises a cluster of diseases caused by different species of protozoa of the genus, *Leishmania.* Leishmaniasis is endemic in many areas of the world, including Brazil, and represents a serious public health problem (WHO, 2007). In Brazil, localized cutaneous leishmaniasis (LCL) is caused mainly by *L. braziliensis* and *L. amazonensis* (Grimaldi et al., 1989). Protection is associated with the development of a T helper-1 (Th1) type cell-mediated immune response (Alexander and Bryson, 2005).

Neuroimmunomodulatory effects have been implicated in leishmaniasis. Stress, gender and age can influence disease outcome in mice and hamsters (Alexander, 1988; Travi et al., 2002; Ruiz et al., 2003; Ehrchen et al., 2004), and hormonal changes have been described in patients infected with *L. mexicana* (Gallindo-Sevilla et al., 2007). Changes in plasma hormone levels have been correlated with an imbalanced cytokine profile in several acute and chronic infections (Reincke et al., 1998; Bhasin et al., 2001; Leal et al., 2003, 2006; Mavoungou et al., 2005; Libonati et al., 2006; Del Rey et al., 2007; Gallindo-Sevilla et al., 2007; Pinto et al., 2007). Hormone level changes have also been implicated in the establishment of human malaria (Kurtis et al., 2001).

Stimulation of neuroendocrine axes, such as hypothalamus–pituitary–adrenal (HPA) and hypothalamus–pituitary–gonads (HPG) induces secretion of hormones which have profound effects on immune response (Besedovsky et al., 1986; Webster et al., 2002). Glucocorticoids (GC) have been recognized as important immunomodulators, promoting a shift from a Th1 to a Th2 cytokine response (Ramírez et al., 1996; Ashwell et al., 2000). DHEA is a

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potential regulator of immune function and counteracts some effects of glucocorticoids (Hazeldine et al., 2010). Estrogens can stimulate antibody production by B cells as well as production of IL-4 and IL-10 (Kanda and Tamaki, 1999; Janele et al., 2006; Straub, 2007). Prolactin and testosterone also produce changes in immune system (Ansar et al., 1985; Olsen and Kovacs, 1996; Brand et al., 2004; Cutolo et al., 2004; Dimitrov et al., 2004).

In the present work, we studied a well-characterized group of male and female LCL patients to investigate hormonal changes in this infection. We also evaluated the relationship between plasma hormone levels and both clinical markers of disease and markers of the immune response.

2. Materials and methods

2.1. Study population

Patients included in this study ($n = 57$) were selected at the Centro de Referência Pirajá da Silva, Jequié (Bahia, Brazil), an endemic area for *L. braziliensis* (de Oliveira et al., 2003). The clinical diagnosis was based on the presence of lesions compatible with LCL, which were confirmed by positive intradermal reaction (DTH test) to the *Leishmania* antigen and, detection of *Leishmania* parasites in biopsy samples after microscopic examination of histological sections or a positive therapeutic test, which was defined as clinical cure following treatment with Glucantime® (20 mg of meglumine antimoniate per kg per day given in cycles of 20 days). Data were collected using a standard protocol chart containing the following information: identification, clinical complaints, physical examination and results of laboratory tests. Exclusion criteria were the following: other pathologies and infections, treatment with hormones or immunosuppressants, alcoholism, pregnancy and amenorrhea. All procedures were approved by the Ethical Committee of Hospital Universitário Edgard Santos – UFBA, BA. Age-matched normal volunteers (NV) living in the same endemic area ($n = 32$; 17 men and 15 women) served as controls for the study. NV had no history of cutaneous lesions characteristic of leishmaniasis and tested negative for the intradermal delayed-type hypersensitivity test (DTH) to the *Leishmania* antigen. When patients were compared with NV we evaluated only the patients age-matched with the controls ($n = 32$; 17 men and 15 women). These 32 patients did not show any difference in clinical and immunological markers when compared to other patients of the study. For analyses of correlations of hormones with cytokines we used all patients.

Clinical evaluation for correlations with hormone or cytokine levels was performed using three parameters: lesion size, time of disease and dose of antimoniate needed to achieve clinical cure. Lesion size was the measurement of the largest diameter of the largest lesion in cm, time of disease was recorded based on patient information and the dose of antimoniate required was based on the number of treatment cycles received by the patient.

2.2. Blood collection and processing

Heparinized peripheral blood was collected between 8 a.m. and 11 a.m., transported on ice to the laboratory and the plasma was stored at −20 °C for measurements of hormone levels.

PBMCs were isolated from heparinized venous blood by passage over a Ficoll Hypaque gradient (Sigma–Aldrich). PBMCs were washed three times and resuspended at a concentration of 5×10^6 cells/mL in RPMI 1640 medium (Gibco, NY) supplemented with 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL) (Gibco, NY) and 10% heat inactivated human AB serum (Sigma–Aldrich). Cells were plated in 24-well tissue culture plates (Costar, Corning Incorporated, NY) at a concentration of 5×10^6 cells/mL and incubated at 37 °C at 5% CO_2.

Stimulation was performed by adding 10 μg/mL of SLA (soluble *Leishmania* antigen). The SLA was prepared as described by Carvalho et al. (1985). Briefly, stationary-phase promastigotes of *L. amazonensis* (MHOMBR86BA-125) were ultrasonicated and centrifuged at 20,000*g* for 2 h. The supernatant was used at a final concentration of 10 μg/mL. PBMC culture supernatants were harvested at 24, 48 and 96 h after in vitro stimulation and maintained at −20 °C until use.

2.3. Hormone assays

The concentrations of cortisol, DHEA-S, estradiol, prolactin and testosterone were determined using automated enzyme immunoassay-based techniques (ACCESS, Beckman Coulter, Fullerton, CA). The detection limits for each hormone measured were the following: cortisol, 0.4 μg/dL; DHEA-S, 2 μg/dL; estradiol, 2 pg/mL; prolactin, 0.25 ng/mL and testosterone, 0.1 ng/dL.

2.4. Cytokine ELISAs

IFN-γ, IL-10 and TNF-α (Pharmingen, San Diego, CA) levels were measured in cell culture supernatants using commercially available ELISA kits. Culture supernatants were harvested at 96 h for IFN-γ, 48 h for IL-10 and 24 h for TNF-α. Assays were performed according to the manufacturer's instructions. The detection limits for each cytokine were as follows: IFN-γ, 55 pg/mL; IL-10, 3 pg/mL and TNF-α, 3 pg/mL.

2.5. Statistical analyses

Hormone concentrations in controls and patients were compared using the Mann–Whitney test. Correlations between the levels of cytokines and hormones were evaluated with the Spearman test. All statistical tests were performed using GraphPad software version 5.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Hormone levels in LCL patients

To determine whether hormone levels were associated with LCL, we assessed plasma levels of cortisol, estradiol, DHEA-S, prolactin and testosterone in NV and LCL patients with an active cutaneous lesion. The clinical profile of the patients analyzed ($n = 57$) is shown in Table 1.

LCL patients ($n = 32$) had lower plasma levels of DHEA-S, prolactin and testosterone than NV ($n = 32$; Fig. 1C, D and F). No difference between patients and controls was observed in levels of cortisol or estradiol (Fig. 1A and B).

Table 1
Clinical data of patients with LCL.

	Males	Females
Number	39	18
Age (years)	29.44 ± 15.38*	37.11 ± 24.75*
Positivity of DTH (%)	78.95	62.50
Lymphadenopathy (%)	60.00	56.20
Location of lesion (%)	59.00 lower limb	66.60 lower limb
Lesion size (cm)	3.53 ± 2.12*	2.73 ± 1.16*
Healing time (days)	78.92 ± 45.57*	68.06 ± 23.19*
Dose (mg/kg)	68.46 ± 37.08*	61.39 ± 28.54*
Number of lesions	1.54 ± 1.16*	1.50 ± 1.15*

* Mean ± standard deviation.

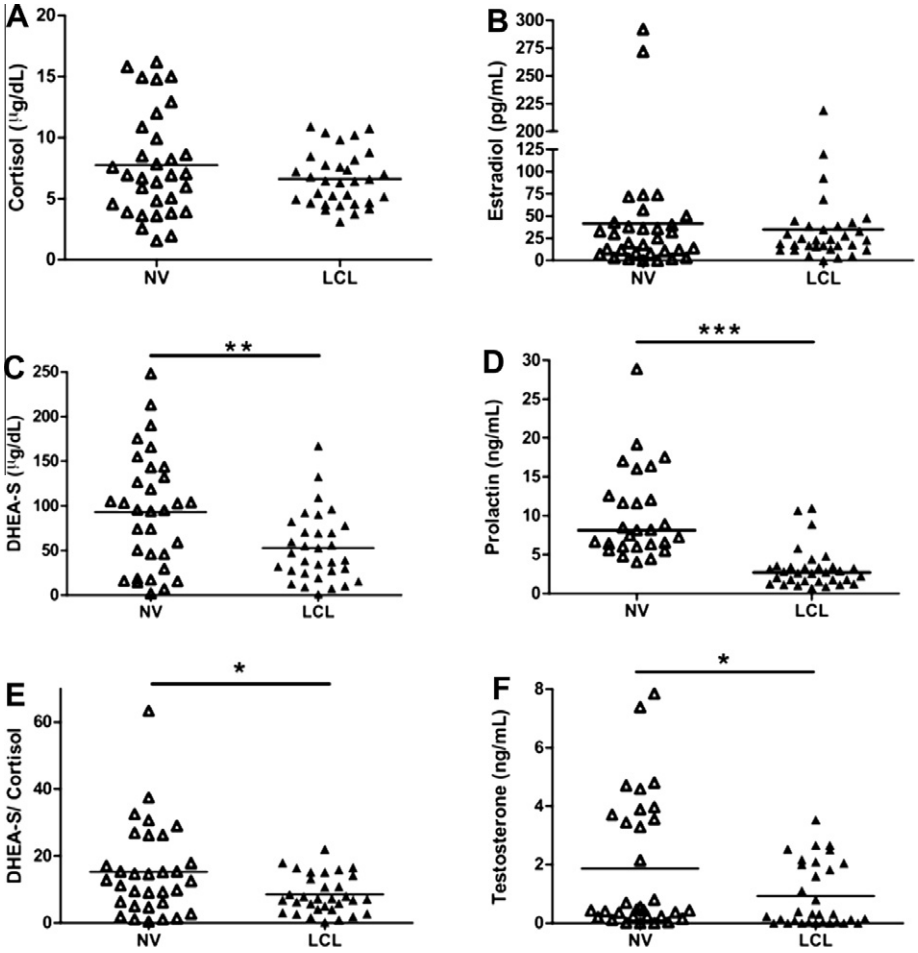

Fig. 1. Plasma hormone levels and ratio of DHEA-S to cortisol in patients with LCL and NV. Patients of both sexes (closed triangles; $n = 32$) were compared with NV of both sexes (open triangles; $n = 32$) by the Mann Whitney test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.2. Cortisol, estradiol and prolactin concentrations correlated with clinical markers and cytokine levels

Possible correlations between hormone levels and clinical or immunological parameters, such as lesion size, healing time, Glucantime dosage and SLA-stimulated IFN-γ, IL-10 and TNF-α levels were analyzed using the Spearman test. We tested the correlation between each hormone and each clinical parameter or cytokine. Cortisol showed a positive correlation with healing time and dose of Glucantime used in the treatment and a negative correlation with in vitro SLA-stimulated IFN-γ levels (Fig. 2A–C). For estradiol, males were analyzed separately from females because of the considerable difference in the concentration of this hormone between the two groups. Plasma levels of estradiol in males correlated positively with lesion size, whereas in females, a correlation was observed with total dose of Glucantime used in treatment (Fig. 3A and B). Prolactin correlated positively with lesion size and negatively with in vitro IFN-γ levels (Fig. 4A and B). Other correlations tested did not reach statistical significance.

3.3. Males and females with LCL have similar changes in hormone levels

To evaluate whether hormone level changes were similar in males and females with LCL, each group was tested separately. Male patients ($n = 17$) showed a reduction in levels of DHEA-S,

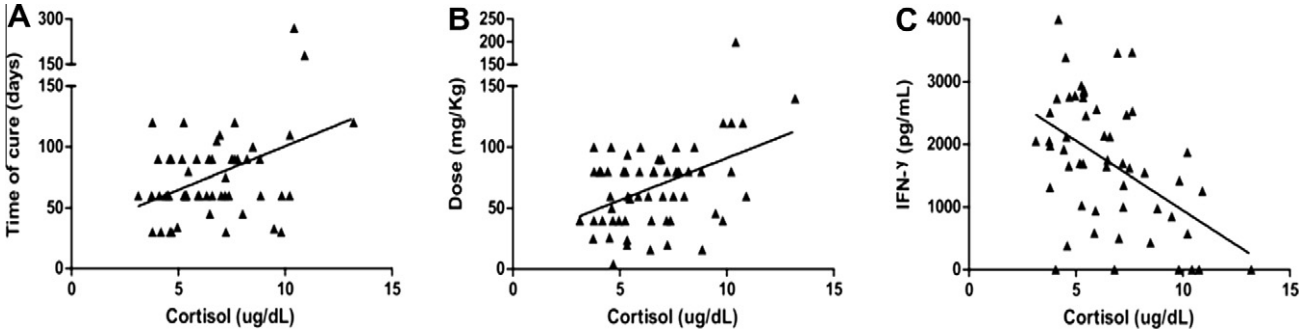

Fig. 2. Correlation between plasma cortisol levels and clinical and immunological markers in LCL patients of both sexes. Cortisol had a positive Spearman correlation with time of cure ($n = 55$; $r = 0.279$; $P = 0.0389$) (A) and dose of glucantime ($n = 57$; $r = 0.3231$; $P = 0.0142$) (B). Cortisol and IFN-γ had a negative correlation ($n = 52$; $r = -0.300$; $P = 0.0357$) (C).

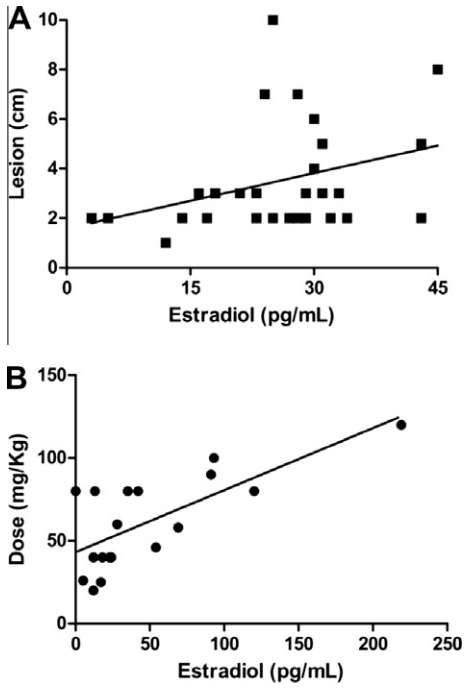

Fig. 3. Correlation between plasma estradiol levels and clinical markers. Estradiol had a positive Spearman correlation with lesion size ($n = 30$; $r = 0.3754$; $P = 0.0403$) in males (A) and dose of glucantime ($n = 18$; $r = 0.6559$; $P = 0.0032$) in females (B).

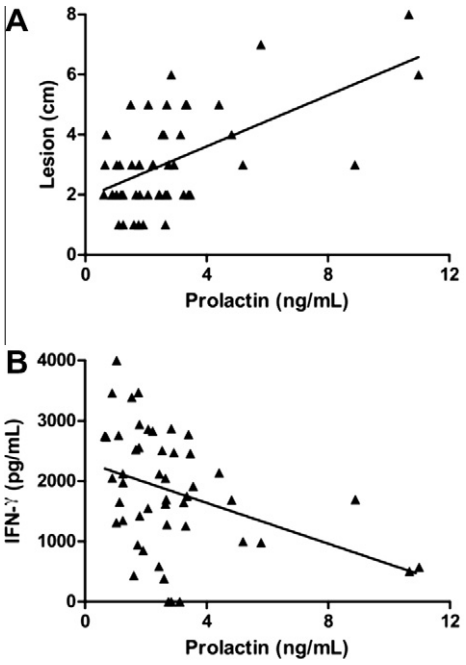

Fig. 4. Correlation between plasma prolactin levels and clinical and immunological markers. Prolactin had a positive Spearman correlation with lesion size ($n = 49$; $r = 0.4508$; $P = 0.0012$) (A) and a negative correlation with IFN-γ levels (supernatant of PBMC cultures) ($n = 49$; $r = -0.3764$; $P = 0.0077$) (B).

prolactin and testosterone compared with controls (Figs. 5C, 6C and E). Female patients ($n = 15$) also had lower levels of prolactin and testosterone compared with controls, but they did show any difference in concentration of DHEA-S (Figs. 5D, 6D and F).

4. Discussion

Immune–endocrine associations have not been sufficiently explored in human leishmaniasis. Herein, we found a reduction in plasma concentrations of DHEA-S, prolactin and testosterone, but not of cortisol and estradiol, in LCL patients. Plasma levels of cortisol, estradiol and prolactin correlated with at least one clinical marker.

There is only one study addressing an immune–endocrine imbalance in human leishmaniasis (Gallindo-Sevilla et al., 2007); this study found lower serum levels of DHEA and cortisol in diffuse cutaneous leishmaniasis (DCL) patients compared with LCL patients or healthy volunteers. When DCL patients were excluded from the study, there was a statistically significant reduction of DHEA in LCL patients compared to age-matched controls. In our study, a decrease in levels of DHEA-S was observed together with reductions in levels of prolactin and testosterone. Concentrations of cortisol and estradiol were similar between LCL patients and NV. These results are consistent with other results described in the literature and indicate that infections do not lead to a standard pattern in neuroendocrine alteration. In some cases, infection induces a reduction and in other cases, infection induces an increase in the same or different hormones.

The endocrine imbalance observed during chronic infections could be the result of the activation of various neuroendocrine axes, such as the HPA axis (hypothalamus–pituitary–adrenal) and the HPG axis (hypothalamus–pituitary–gonads) by the immune system (Besedovsky et al., 1986; Webster et al., 2002). Some cytokines, especially IL-1, IL-6 and TNF-α, can act directly on the central nervous system (CNS), resulting in the activation of neuroendocrine axes, mainly the HPA axis (Berkenbosch et al., 1987; Holsboer et al., 1988; Naitoh et al., 1988; Sharp et al., 1989), and hormones can influence cytokine production (Besedovsky et al., 1986). Moreover, in some types of infections, the presence of microorganisms in the glands can affect hormone secretion (Reincke et al., 1998; Corrêa-de-Santana et al., 2006). In LCL, parasites are present almost exclusively in the skin and draining lymph nodes (de Moura et al., 2005); therefore, the endocrine imbalance seen in LCL is unlikely to be caused by the direct presence of the parasite but instead, may be due to the action of cytokines in the CNS or glands.

Plasma concentrations of some hormones evaluated in this study correlated with clinical and/or immunological parameters. IFN-γ is the hallmark cytokine of a Th1 immune response and is strongly linked to protection against leishmaniasis. Cortisol showed a positive correlation with healing time and dose of Glucantime used in the treatment and a negative correlation with levels of IFN-γ. One of the major actions of glucocorticoids is to promote a shift from a Th1 to a Th2 cytokine response (Ramírez et al., 1996; Ashwell et al., 2000). In LCL, the balanced production of Th1 and Th2 cytokines promotes the healing of lesions because as overproduction of IFN-γ could lead to more severe tissue destruction, while high levels of anti-inflammatory cytokines could impair parasite clearance (Boom et al., 1990; Ajdary et al., 2000; Alexander and Bryson, 2005). Studies have reported both the reactivation of cutaneous and visceral leishmaniasis after glucocorticoid treatment in humans and mice (Rousseau et al., 1998; Pittalis et al., 2006; Tuon et al., 2007) and an unusual disseminated mucocutaneous leishmaniasis resulting from chronic use of glucocorticoids (Motta et al., 2003). A decreased ratio of DHEA-S to cortisol was observed in LCL patients in our study, and this also favors the development of a Th2 response. DHEA-S is a precursor of DHEA and no biological function has been ascribed to it besides being a precursor of DHEA (Hazeldine et al., 2010). The long half-life of plasma DHEA-S coupled with the limited diurnal variation make DHEA-S a convenient marker for the assessment of adrenal production. DHEA is a potential regulator of

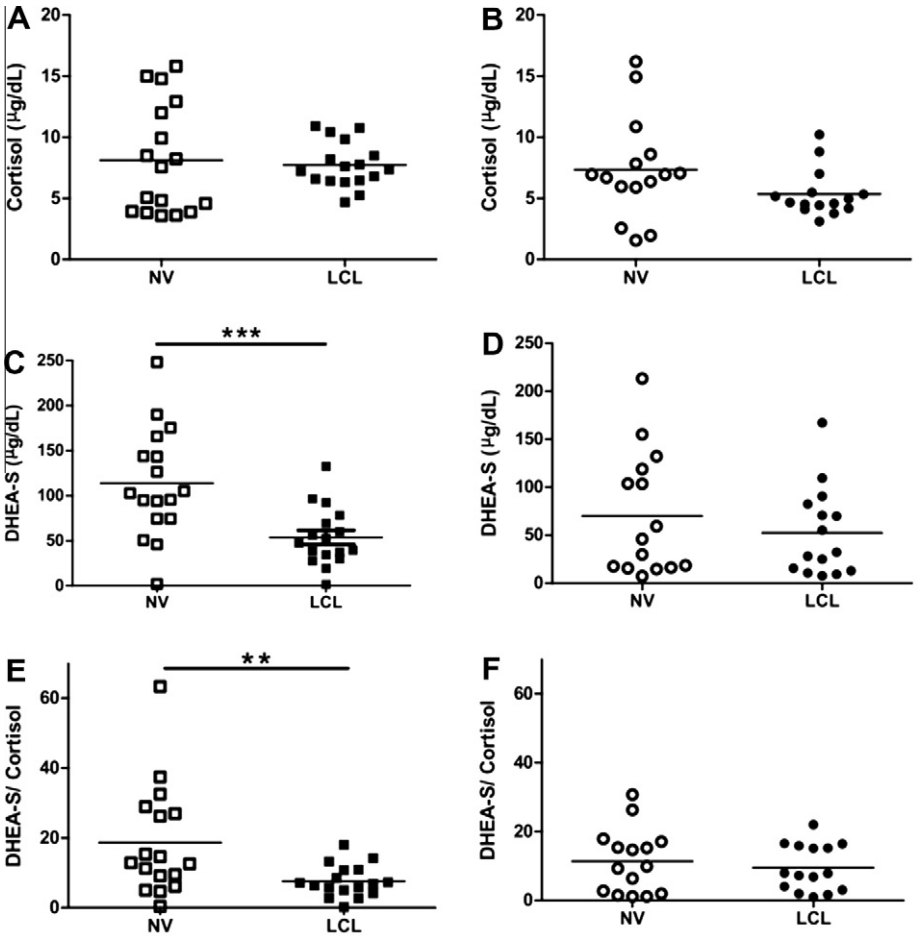

Fig. 5. Plasma hormone levels and ratio of DHEA-S to cortisol in males and females with LCL and NV. Male patients (square) ($n = 17$) and females patients (circle) ($n = 15$) were compared with NV by the Mann Whitney test. $^{**}P < 0.01$, $^{***}P < 0.001$.

immune function and counteracts some effects of glucocorticoids (Hazeldine et al., 2010). This hormone can stimulate the IL-2 secretion by T cells and inhibit IL-6 and IL-10 production (Suzuki et al., 1991; Spencer et al., 1996; Straub et al., 1998). Thus, in LCL, the HPA axis could be involved in maintenance of a Th2 response and restriction of the Th1 response.

Plasma levels of estradiol correlated positively with other important clinical parameters, such as size of the lesion in males and dose of Glucantime used in treatment in females. Estrogens exhibit several effects on the immune response, some of which could influence LCL development. Estrogens can stimulate antibody production by B cells as well as production of IL-4 and IL-10 (Kanda and Tamaki, 1999; Janele et al., 2006; Straub, 2007). In experimental models of leishmaniasis, antibodies were not protective and may have enhanced susceptibility to infection (Kima et al., 2000). IL-4 inhibited IFN-γ production and macrophage activation in experimental models, and IL-10 and other Th2 cytokines led to disease exacerbation (Boom et al., 1990; Ajdary et al., 2000; Alexander and Bryson, 2005). Considering such mechanisms, it is possible that estradiol is involved in lesion development in leishmaniasis.

Prolactin positively correlated with lesion size and negatively correlated with IFN-γ levels. IFN-γ and TNF-α can inhibit prolactin secretion by the anterior pituitary (Walton and Cronin, 1990), and this could explain the reduction in prolactin levels in individuals with LCL as these cytokines were elevated in LCL patients. Although some authors have associated the stimulatory effect of prolactin with the release of pro-inflammatory cytokines, such as TNF-α, IL-2, IFN-γ and IL-12 (Brand et al., 2004; Dimitrov et al., 2004), our results showed a negative correlation between levels of prolactin and IFN-γ. Inhibition of IFN-γ production could result in increased lesion size because this cytokine is necessary for macrophage activation and parasite elimination.

Sexual dimorphism in the immune response has been noted by many authors (Ansar et al., 1985; Olsen and Kovacs, 1996). Females exhibit more vigorous humoral responses and a greater tendency to develop autoimmune disease than males (Butterworth et al., 1967; Ansar et al., 1985; Klein, 2004). We examined the possibility that the endocrine changes observed in LCL were detected only in males or females. Our results indicated that hormonal changes were similar between the sexes, except for DHEA-S. Levels of DHEA-S were the same in patients from both sexes, but when patients were compared with NV, the reduction was more marked in males than females. This may be due to the fact that healthy male volunteers had more elevated baseline concentrations of DHEA-S than healthy female volunteers.

Our results indicate that in LCL, neuroendocrine regulation could restrict Th1 responses by reducing DHEA-S and prolactin levels and ratio of DHEA-S to cortisol. Although the Th1 response is necessary for the elimination of parasites, the overproduction of IFN-γ and TNF-α would be harmful to the host, as high levels of these cytokines could increase damage to tissue. The decrease in plasma testosterone levels detected in LCL patients could also contribute to host defense mechanisms as this hormone is associated with an increased susceptibility to many parasitic infections, including experimental leishmaniasis (Mock and Nacy, 1988; Klein, 2004). Testosterone exhibits several immunosuppressive effects, such as

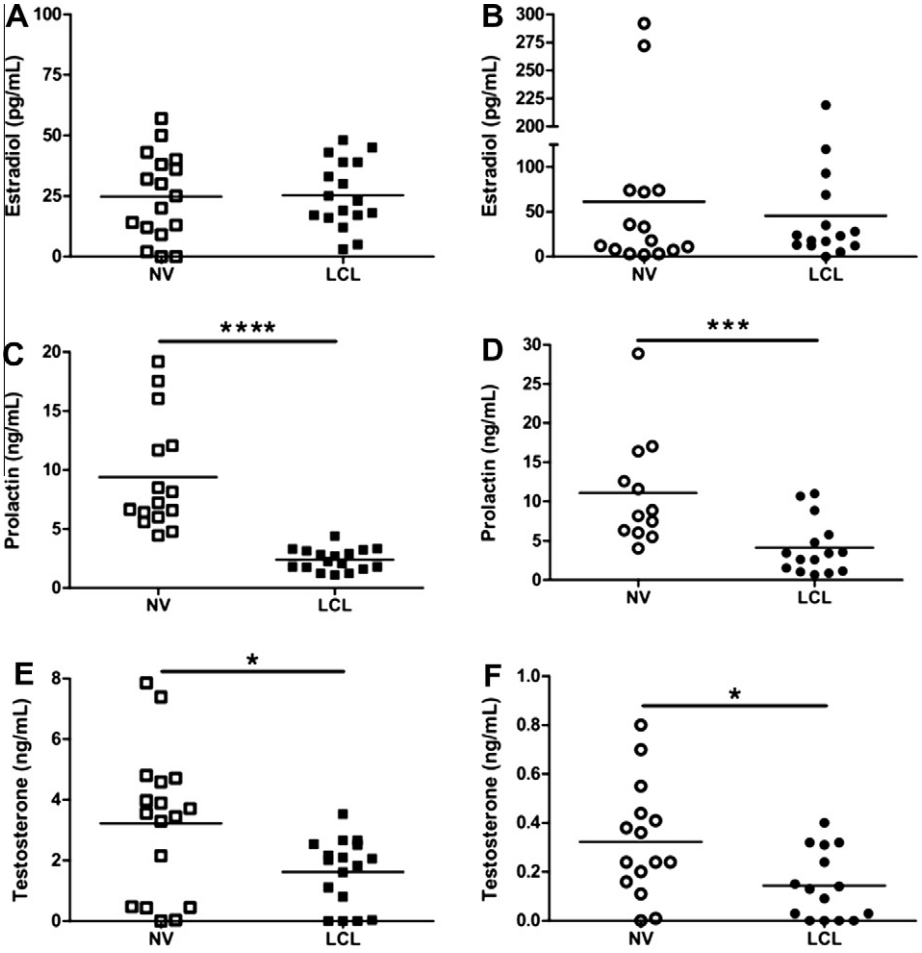

Fig. 6. Plasma hormone levels in males and females with LCL and NV. Male patients (square) ($n = 17$) and female patients (circle) ($n = 15$) were compared with NV by the Mann Whitney test. $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

promoting an increase in infection of macrophages by *L. donovani* (Zhang et al., 2001; Klein, 2004). Although the estradiol levels in LCL patients were similar to those in the NV, there is a possibility that the reduction in testosterone levels could result in its conversion to estradiol because inflammatory cytokines have been found to stimulate aromatase activity (Cutolo et al., 2004). In conclusion our results indicate that patients with LCL can exhibit an immune–endocrine imbalance with reduction of plasma levels of DHEA-S, prolactin and testosterone. The endocrine–immune interactions can play an important role in LCL as the levels of some hormones correlate with cytokine levels and clinical markers. The present study provides new insights into the regulation of the immune response in leishmaniasis. The neuroimmunomodulation observed in LCL patients appears to be beneficial to the host and contributes to the healing of lesions. Perhaps more aggressive forms of leishmaniasis may be the result of changes in neuroendocrine regulation. Knowledge of the endocrine mechanisms involved in regulating the immune response in LCL could be important for development of pharmacological alternatives for treatment of this disease.

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