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Treatment with dehydroepiandrosterone in vivo and in vitro inhibits reproduction, growth and viability of *Taenia crassiceps* metacestodes

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Abstract

The aim of this work was to explore the effect of dehydroepiandrosterone (DHEA) on the establishment, growth and reproduction of the metacestode stage of the tapeworm *Taenia crassiceps*, both in vivo and in vitro. Administration of DHEA prior to infection in mice of both sexes reduced the parasite load by 50% compared with untreated mice. This protective effect was not associated with the immune response, since there was no effect of DHEA treatment on mRNA levels of IL-2, IFN-γ, IL-4 or IL-10. DHEA treatment of infected mice increased androgen receptor expression in splenocytes of both sexes. Moreover, in vitro treatment of *T. crassiceps* with DHEA reduced reproduction, motility and viability in a dose- and time-dependent fashion. Results indicate that DHEA has strong negative direct modulatory effects on murine cysticercosis. We suggest the use of hormonal-analogues for protective purposes as a therapeutic approach to prevent murine cysticercosis.

Keywords: DHEA; *Taenia crassiceps*; Parasite; Infection; Hormones; Cysticercosis

1. Introduction

The host–parasite interaction in human neurocysticercosis caused by *Taenia solium*, as well as in porcine (*T. solium*) and murine cysticercosis (*Taenia crassiceps*), is extremely complex and the immune response has traditionally been considered paramount in controlling the infection (Sciutto et al., 1991). Paradoxically, the immune response has also been considered a partial cause of the natural disease in humans. The outcome of the infection depends on a balance between the host immune response and the endocrinological environment, which varies among races of pigs or mouse strains (Larralde et al., 1995; Morales-Montor and Larralde, 2005). Many details of this interplay remain to be elucidated.

Recent studies have cited an interaction between the neuroendocrine and the immune systems in the regulation of anti-parasite responses (Klein, 2004). For instance, it has been shown that the hypothalamic–pituitary–adrenal axis modulates the immune response (Morales-Montor et al., 2001a), whereas the hypothalamic–pituitary–gonadal axis is sensitive to it (Morales et al., 1996). Hormones produced by both axes are involved in the regulation of host–parasite interactions, particularly in schistosomiasis (Eloi-Santos et al., 1992), cysticercosis (Morales et al., 1996), trypanosomiasis (do Prado et al., 1998) and amebiasis (Acuna-Soto et al., 2000). In experimental cysticercosis by *T. crassiceps*, it is well known that androgens play a significant role in down-regulating the parasite load in male and female Balbc/AnN mice (Morales-Montor and Larralde, 2005). Nevertheless, studies with other non-gonadal-androgenic-hormones have not been performed.

Dehydroepiandrosterone (DHEA) is a steroid hormone produced from cholesterol by the adrenal glands, the

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gonads, adipose tissue and the brain. It is the most abundant hormone in the human body. In blood, most DHEA is found as DHEA sulphate (DHEAs), with levels of the sulphate form being approximately 300 times higher than free DHEA. In humans, and in mammals generally, DHEA is the dominant steroid hormone and precursor of sex steroids and has proved to be an important molecule in resistance against a variety of infections. These infections include intracellular parasites such as *Plasmodium falciparum* and *Plasmodium berghei* (Freilich et al., 2000), *Cryptosporidium parvum* (Rasmussen and Healey, 1992), as well as extracellular parasites [*Entameba histolytica* (Carrero et al., 2006), *Schistosoma mansoni* (Morales-Montor et al., 2001); *Trypanosoma cruzi* (dos Santos et al., 2005)], although the molecular mechanisms by which DHEA has such widespread parasiticidal effects are still being discussed.

The immunological mechanisms associated with DHEA protection are not well known. On the contrary, DHEA has been shown to have a direct parasiticidal effect. For instance, in vitro, DHEA treatment resulted in a decrease in the growth and viability of *E. hystolitica* trophozoites, and its effect was presumably due to the inhibition of glucose-6-phosphate dehydrogenase (G6PD) enzyme activity (Carrero et al., 2006).

The goal of the present study was to investigate whether DHEA has direct in vitro and in vivo immune-system modulating effects on *T. crassiceps* reproduction, growth, viability and infectivity. Our results suggest that DHEA treatment may be used as a new therapeutic approach against both experimental and natural cysticercosis.

2. Materials and methods

2.1. Reagents

Culture grade human DHEA was purchased from Sigma, St. Louis, MO. DHEA was dissolved in ethanol to the desired stock concentration and sterilised by passage through a 0.2-mm millipore filter. All other reagents were purchased from common commercial sources.

2.2. Parasites and experimental infections

A new (ORF) strain of *T. crassiceps*, donated by R. Kuhn, was used in all experiments. Parasites were maintained in female BALB/c mice by sequential i.p. inoculation of the metacestodes. Metacestodes for experimental infections were obtained from female donor mice infected 3–6 months earlier. Twenty small (approximately 2-mm in diameter) non-budding *T. crassiceps* larvae were suspended in 0.6 ml PBS (0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2) and injected i.p. into 8-week-old mice. Mice were caged in groups of five in a common room under controlled temperature and 14 h dark/10 h light cycle in the animal facility of the Biological Sciences Building at the Institute of Biomedical Research (IIB), and were inspected by the IIB Animal Care and Use Committee and Governmental agencies to ensure compliance with federal regulations and international guidelines. They were fed Purina Diet 5015 (PMI Nutrition International, Brentwood, MO) and water ad libitum, and were sacrificed at 8 weeks of infection by cervical dislocation subsequent to ether anaesthesia. After death, parasites were visually counted in each mouse by collecting the cysts after thoroughly rinsing the peritoneal cavity with PBS. Parasites were never found outside the peritoneal cavity.

2.3. DHEA administration

DHEA was injected sub-dermally (200 μg/25 g), every other day, during the 8 weeks of infection, starting 1 week prior to infection. The vehicle in which the DHEA was diluted (mineral oil) was also administered to another group of infected mice. After 1 week, mice were infected as described above and killed 8 weeks after infection.

2.4. DHEA measurements

Blood for steroid determinations was collected by cardiac puncture performed in deeply anaesthetised mice. Steroids were ether-extracted and solubilised in the buffer used for the immunoassay. The DHEA serum concentration was determined by liquid-phase kinetics enzyme immunoassay kits (D.S. Labs), according to the manufacturer's instructions (www.dslabs.com). After reactions were developed, the samples were read at 450 nm in an ELISA reader.

2.5. IL-2, IFN-γ, IL-4, IL-10 and androgen receptor (AR) expression

Total RNA from spleen and testicle of uninfected, placebo-treated or DHEA-treated mice 8 weeks p.i. was reverse transcribed, followed by specific PCR amplification of IL-2, IFN-γ, IL-4, IL-10, androgen receptor (AR) and β-actin genes. Primers used for amplification have been previously described (Vargas-Villavicencio et al., 2005). Total RNA (5 μg) from each tissue was incubated at 37 °C for 1 h with 400 units of M-MLV reverse transcriptase (Applied Biosystems, Boston, MA) in 1.25 μg of reaction volume containing 50 mM each of dNTP and 0.05 μg oligo (dt) primer (Gibco, NY). cDNA reaction (5 μl) was subjected to PCR in order to amplify specific sequences of the specified genes. The 50 μl PCR included 5 μl of previously synthesised cDNA, 25 μl of 10× PCR-buffer (Biotecnologías Universitarias, México), 1 mM MgCl, 0.2 mM of dNTP, 0.05 μM of each primer and 2.5 units of Taq DNA polymerase (Biotecnologías Universitarias, México). Total PCR (20 μl) products of each sample was electrophoresed on 2% agarose gel. PCR products were visualised by staining with ethidium bromide. A single band was detected in all cases, as expected. In order to determine if all amplified genes as well as the constitutively expressed

uninfected gene (β-actin) were in the exponential phase of amplification, and to make sure that changes in expression were not due to artifact (such as β-actin being in the stationary phase), we obtained the RNA, cycling and temperature curves for each analysed gene.

2.6. Densitometric analysis

Hybridisation signals were quantified by densitometric scanning of multiple autoradiograms of several exposures, and were represented as the relative expression, which is the ratio of the signal of the amplified genes relative to the expression of the β-actin gene, a constitutively expressed gene used as an internal control of expression.

2.7. In vitro DHEA assays

Culture grade DHEA was obtained from Sigma. For in vitro tests, it was dissolved in AIM-V (free of calf serum and other hormones) culture medium to the desired stock concentration and sterilised by passage through a 0.2-mm millipore filter. The experimental design was as follows: using a 24-well culture plate, six wells were used for untreated trials, six wells were supplemented with the vehicle in which DHEA was diluted, six wells were treated with three different concentrations of DHEA. Concentrations of DHEA were randomised across the plates. Control cysticerci were treated with the solvent in which DHEA was diluted, so that a constant volume of solvent (2 ml) was added to each well. Reproduction was measured as the number of buds that each cyst produced in response to treatment and they were counted directly under an inverted microscope (Olympus, MO21) at 10× and 100× magnifications.

2.8. Statistical analysis

We used a three factorial experiment. Independent variables were: (1) treatment: (two levels: DHEA or vehicle); (2) gender (two levels: female, male); (3) infection (two levels: Yes, No). Dependent variables were the number of parasites, serum sex-steroid concentrations and the expression of IL-2, IFN-γ, IL-4, IL-10 and AR in the tissue sample, measured by the OD of the corresponding gel divided by the OD of β-actin in the same tissue sample in the same gel, which was used as the control gene in the amplification technology. Two experiments were performed ($n = 5$ mice in each treatment), and data were analysed using one-way analysis of variance (ANOVA). When performed, post hoc individual contrasts of group means to test for significant differences were carried out using *t*-tests. Hormone dose–response time curves were estimated in three independent experiments performed with freshly isolated *T. crassiceps* cysticerci. DHEA was tested at five different doses; each dose was run in triplicate. The response variable used for statistical analysis was the number of cysticerci for each hormone dose and the time of exposure in each experiment. Differences between groups were estimated using the ANOVA test. Differences were considered significant when $P < 0.01$. The software Prism 2.01 (GraphPad Software Inc.) was used to calculate probability values.

3. Results

3.1. Parasite loads

Due to the extremely variable parasite loads found in murine *T. crassiceps* cysticercosis, linked to a range of biological factors of the parasite and the host, we decided to plot the individual parasite burdens found in each mouse after each treatment. Fig. 1 shows the individual parasite loads recovered from the peritoneal cavity of mice of both sexes. In control mice, females were more susceptible to infection ($P < 0.001$) than male mice. DHEA treatment decreased parasite loads to a similar degree in females, (50%, $P < 0.001$), and males (40%, $P < 0.001$). Treatment with the vehicle did not affect parasite numbers in females or in males; they remained similar to those in control groups (Fig. 1).

3.2. DHEA serum levels

Before starting DHEA treatment in mice, we evaluated DHEA serum levels during the time course of the infection (4, 8, 16 weeks) in uninfected and infected female and male mice (Fig. 2), as previously shown (Morales-Montor et al., 2001). We made an ether extraction of all serum steroids, concentrated those, and used a specific commercial kit to detect DHEA in serum. Fig. 2 shows that, as infection advances, there is a reduction (50%) in DHEA serum levels in 16-week infected mice ($P < 0.001$) compared with uninfected mice of both sexes. Female mice had higher DHEA serum levels than male mice ($P < 0.001$).

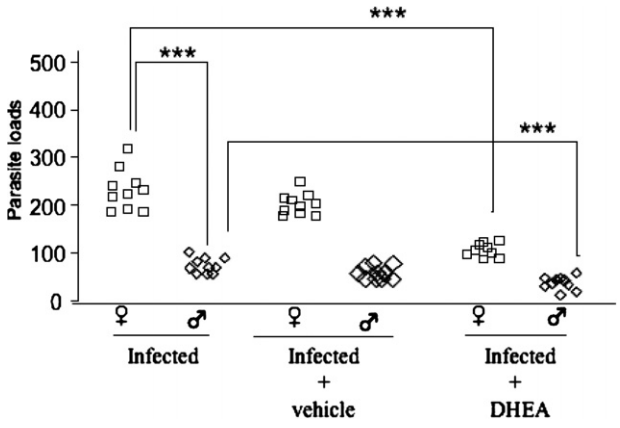

Fig. 1. Effect of dehydroepiandrosterone (DHEA) administration on parasite load. Data show the number of parasites recovered from the peritoneal cavity of 10 female and 10 male BALB/c mice at 8 weeks p.i. Each point represents individual parasite loads. $^{***}P < 0.001$ compared with both sexes mice and treated-group.

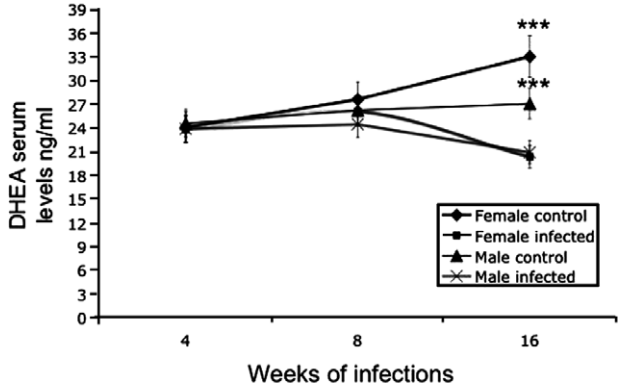

Fig. 2. Dehydroepiandrosterone (DHEA) serum levels throughout the course of the infection in individual mice of both genders with different treatments. Each serum sample was determined in duplicate for each mouse. $^{***}P < 0.001$ compared with uninfected mice of both genders.

3.3. Th-1 and Th-2 immunity

Data obtained for the Th1-type immunity mRNA cytokine profile showed that uninfected (control) male mice expressed higher IL-2 levels (two-fold) than control female mice (Fig. 3). However, DHEA treatment in uninfected female mice changed the pattern of expression of IL-2, which was enhanced 3.5-fold, while in males there was no effect.

DHEA treatment of infected mice of both sexes had no effect on IL-2 mRNA expression. With respect to IFN-γ expression, no statistical differences were found between any of the analysed groups, though there was a clear sex-associated expression that was not affected by DHEA treatment. Vehicle treatment had no effect whatsoever on the cellular immune response of infected animals (Fig. 3). Fig. 3 also shows the relative mRNA expression of IL-4 and IL-10 obtained in splenocytes from mice of both genders in response to different treatments. IL-4 and IL-10 production were not different in any of the groups studied. Vehicle treatment did not affect IL-4 or IL-10 production (Fig. 3).

3.4. AR expression

AR expression was studied to try to explain the lack of effect of DHEA on the measured immune parameters (Fig. 4).

The spleens of infected and DHEA-treated female and male mice showed a two-fold increase in AR mRNA content ($P < 0.001$) compared with uninfected, uninfected DHEA-treated, and vehicle-treated mice. The AR expres-

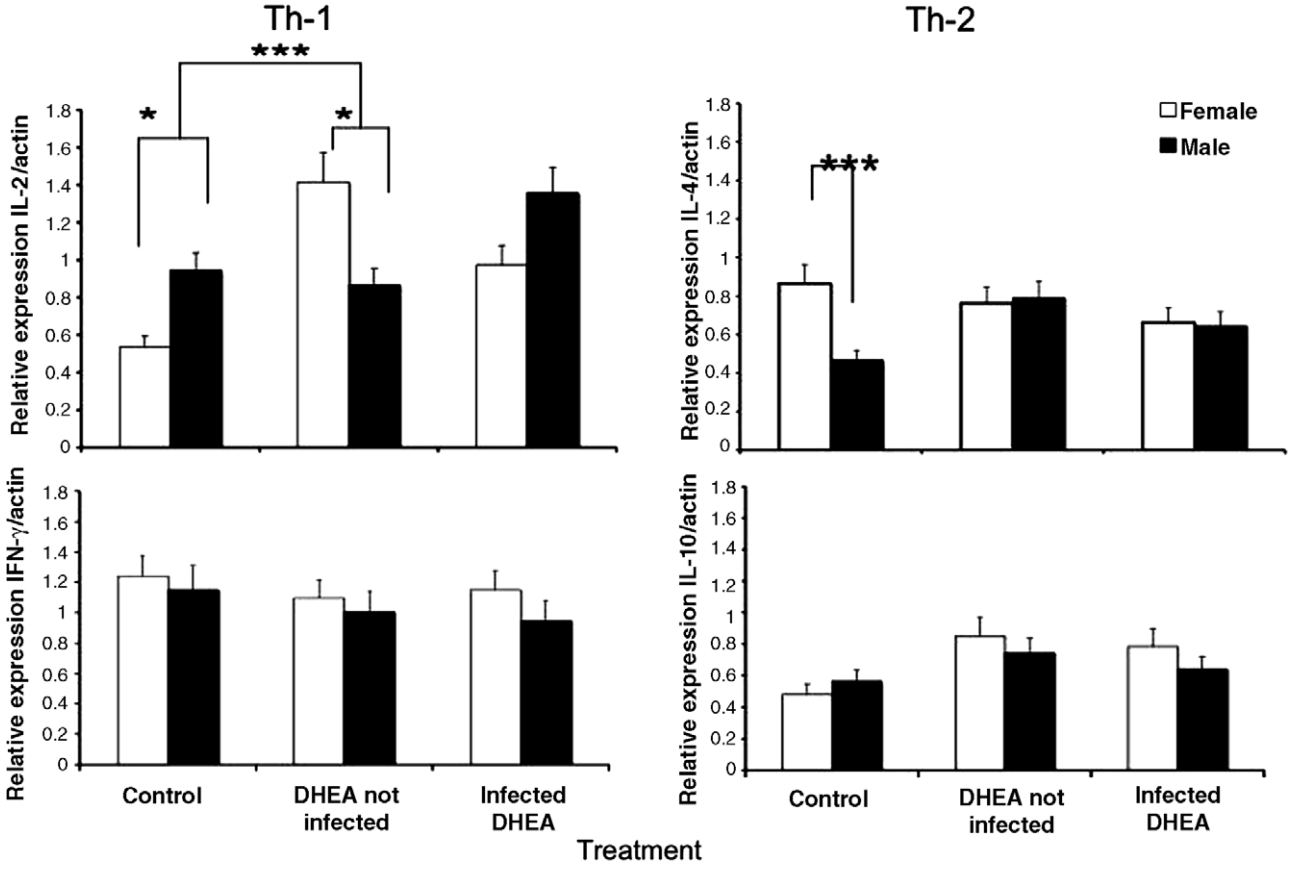

Fig. 3. Effect of chronic infection and dehydroepiandrosterone (DHEA) treatment in the expression of Th-1 (IL-2 and IFN-γ) and Th-2 (IL-4 and IL-10) in splenocytes of mice of both genders infected with *Taenia crassiceps* cysticerci. Data are presented as mean ± SD of two different experiments ($n = 5$). Each splenocyte culture was done in triplicate, after an 8 week infection period. $^{*}P < 0.05$; $^{***}P < 0.001$; both compared with the control group.

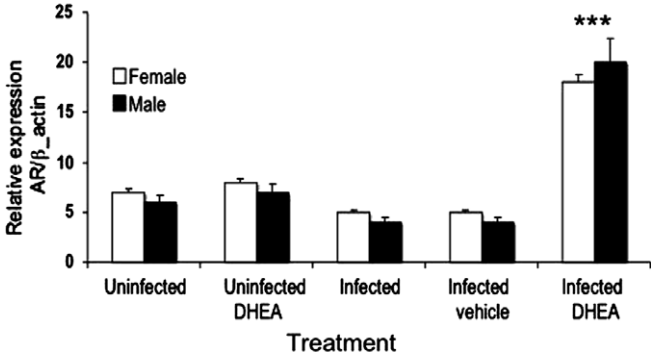

Fig. 4. Androgen receptor (AR) and β gene expression during infection with *Taenia crassiceps*. The results of gene expression are reported as densitometric data of the autoradiographic signal. The relative expression was obtained by correcting the expression of AR to that of β-actin. Data are from five mice, and each experiment was duplicated. Values are mean ± SD. $^{***}P < 0.001$ when compared with the uninfected group. DHEA, dehydroepiandrosterone.

sion level in the spleen of infected mice of both genders was as high as that found in the testicles of uninfected male mice, which was used as positive control tissue of high AR expression (Fig. 4). The expression level of the constitutive β-actin gene was constant in all examined tissues.

3.5. In vitro assays

In vitro DHEA treatment of cysticerci had an inhibitory effect on the parasite, at either physiological or pharmacological doses of DHEA (Fig. 5). DHEA treatment also affected cysticercus motility and survival (data not shown). The effect was also time-dependent, reaching a plateau after 10 days of culture with a sub-lethal dose of DHEA (Fig. 5).

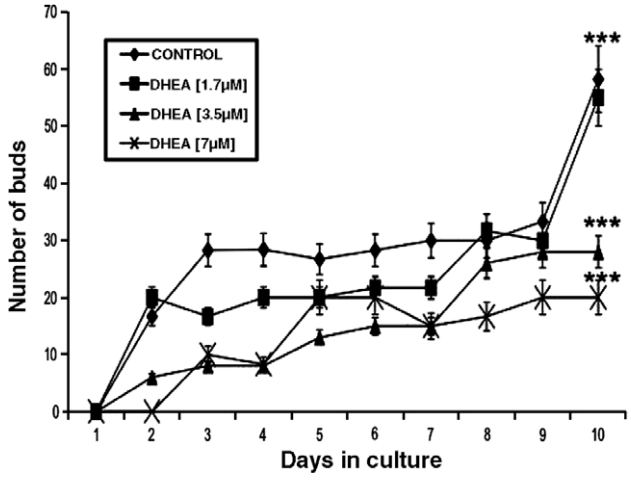

Fig. 5. Dose–response and time curves of *Taenia crassiceps* cysticercus reproduction after dehydroepiandrosterone (DHEA) exposure. Ten cysticerci were incubated for 10 days with different concentrations of DHEA. Each point represents mean (±SD) of five assays counting the number of buds in each parasite and viability in each cultured well. Data were pooled. $^{***}P = 0.01$ with respect to dose-response and time curves after DHEA exposure.

4. Discussion

In another study, exogenous DHEA administration was shown to up-regulate the immune system, specifically the cellular immune response, by increasing the natural killer cell number and function (Loria and Padgett, 1998). Our present findings do not support this notion, since IL-2 mRNA levels do not change in response to DHEA treatment (not shown). The lack of effect of DHEA on cytokine mRNA, but its dramatic effect *in vivo* on parasite load and parasite reproduction, and *in vitro* on survival, support the hypothesis that DHEA exerts its protective properties via direct effects on the parasite. To the best of our knowledge, this effect is consistent with the known effects of DHEA on the survival of other parasites, both metazoan (Fallon et al., 1998; Morales-Montor et al., 2001a) and protozoan (Carrero et al., 2006).

For instance, it has been suggested that in human schistosomiasis, DHEA is the cause of the puberty-associated drop in susceptibility (Fulford et al., 1998). This idea has been reinforced by experiments in which treatment of mice with the bloodstream form of DHEA, DHEA-s, protected them from infection with *S. mansoni* (Fallon et al., 1998). We here extend these findings to the role of DHEA in the protection of mice against *T. crassiceps* infection.

Our findings in mice of a decrease in DHEA levels as infection progresses agree with previous results in a *S. mansoni*-baboon model, in which baboons with primary infections showed decreasing levels of DHEA as the infection progressed, compared with uninfected and re-exposed baboons (Morales-Montor et al., 2001).

The protective effect of DHEA has also been demonstrated in other parasitic infections. Exogenous DHEA administration is able to increase the levels of lytic antibodies and to reduce *T. cruzi* parasitemia in rats (dos Santos et al., 2005). DHEA treatment of mice infected with the protozoan parasite *C. parvum* significantly reduced both the shedding of fecal oocysts and parasite colonisation of the ileum (Rasmussen et al., 1993, 1995). Our results showing that DHEA treatment protects mice against *T. crassiceps* infection support and extend the notion that androgens are an important factor involved in limiting *T. crassiceps* establishment in immunocompetent hosts. Previous immunological experiments have suggested that testosterone and dihydrotestosterone, two potent androgens (such as DHEA), negatively regulate parasite reproduction in mice of both sexes, presumably by interfering with the thymus-dependent cellular immune mechanisms that inhibit parasite growth (Th-2) and enhancing those that facilitate it (Th-1) (Morales et al., 1996; Morales-Montor et al., 2001b), but also by directly affecting parasite motility, survival and reproduction (Escobedo et al., 2004).

Based on the idea that the molecular mechanisms by which gonadal or adrenal steroids (such as DHEA) affect immune system function may be due to their interaction with a specific nuclear receptor, we decided to amplify the classic AR in the spleen of all experimental animals. We showed

that DHEA treatment of infected mice of both genders showed up-regulation of AR expression. These findings suggest that DHEA probably acts through mechanisms that involve a classical nuclear receptor in the immune system, though in our present experiments there was no effect of DHEA on immune stimulation in infected mice.

However, since we did not find a regulatory effect of DHEA on the host immune response, the direct effect of DHEA on parasites was considered. For instance, it has previously been demonstrated that in vitro treatment of cercariae, schistosomula and adult *S. mansoni* with DHEA strongly affect parasite survival (Morales-Montor et al., 2001a). The same study showed that mechanically transformed schistosomula were far more susceptible to the effects of DHEA than schistosomula recovered from mice. Interestingly, adult male worms were considerably less sensitive than females to the lethal action of DHEA, but when adult worms were paired, attrition was markedly reduced. DHEA also significantly inhibited oviposition in vitro (Morales-Montor et al., 2001a).

In vitro, DHEA treatment of *E. histolytica* trophozoites also reduced the growth and viability of this parasite. The effects of DHEA were associated with the inhibition of G6PD activity (Carrero et al., 2006; Di Monaco et al., 1997). Also, DHEA is known to exert anti-malarial protection, via the enhanced opsonisation and phagocytosis of rings, the early forms of this parasite (Ayi et al., 2002; Safeukui et al., 2004). Our results confirm and extend the notion that DHEA is a strong parasiticidal agent, since in vitro DHEA treatment of *T. crassiceps* remarkably reduced the reproduction rate and viability of cysticerci. Also, in our present experiments, the effects of DHEA significantly reduced the parasite burden to a similar degree in males and females. Finally, our results support and extend the notion that DHEA is a potentially useful treatment against a large variety of parasitic diseases. The fact that DHEA interferes with the development of *T. crassiceps* cysticerci may be applied to the development of future therapeutic protocols against other cysticercal infections, particularly those affecting pigs and humans.

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