Coccidial infection in immunosuppressed mice: prophylaxis and treatment with dehydroepiandrosterone

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العدوى الكُرَوَانية (الكوكسيدية) في الفئران المكبوتة المناعة: اتقاؤها ومعالجتها بمادة ديهيدرو إيبي آندروستيرون

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خلاصة: إن داء خفيّات الأبواغ وداء المقوّسات، مرضان انتهازيان تسبّبهما طفيليات كُرَوَانية يمكن أن تودي إلى عدوى تهدّد حياة المرضى المكبوتي المناعة. وتهدف هذه الدراسة إلى تقييم المفعول الوقائي والعلاجي لمادة ديهيدرو إيبي آندروستيرون (DHEA) في الفئران المكبوتة المناعة والمصابة بعدوى خفيّات الأبواغ الصغيرة والمقوّسات القندية العديمة الفوعة. وقد نُقلت إلى الفئران عدوى بالكيسات البيضية لخفيّات الأبواغ أو بكيسات المقوّسات. وتم التقييم من خلال معدلات النفوق وعدّ الطفيليات والفحوص المجهرية الإلكترونية. ووُجد أن معدلات النفوق قد انخفضت بدرجة يعتد بها في سائر المجموعات المعالجة. كما لوحظ انخفاض يعتد به في عدّ الكيسات البيضية لخفيّات الأبواغ في البراز والزغابات المعوية، وكذلك في كيسات المقوّسات في أدمغة الفئران المصابة بالعدوى في كل المجموعات. وكان الدواء أكثر فاعلية عندما أعطي للفئران قبل إصابتها بالعدوى.

ABSTRACT Cryptosporidiosis and toxoplasmosis are diseases caused by opportunistic coccidial parasites that can lead to life-threatening infection in immunocompromised patients. We evaluated dehydroepiandrosterone as prophylaxis and therapy in immunosuppressed mice infected with *Cryptosporidium parvum* and avirulent *Toxoplasma gondii*. Mice were infected with either *Cryptosporidium* oocysts or *Toxoplasma* cysts. Assessment was by mortality rates, parasitic counts and electron microscopic studies. Mortality rates were significantly reduced in all treated groups. A significant reduction in the cryptosporidial oocyst count in stool and intestinal villi and in *Toxoplasma* cysts in the brains of infected mice was observed in all the groups. The effect of the drug was greater when given prior to infection.

L'infection coccidienne chez les souris immunodéprimées: prophylaxie et traitement par déhydroépiandrostérone

RESUME La cryptosporidiose et la toxoplasmose sont des maladies causées par des parasites coccidiens opportunistes qui peuvent causer une infection mettant en danger la vie des patients qui présentent un déficit immunitaire. Cette étude évalue la déhydroépiandrostérone en tant que prophylaxie et thérapie chez des souris immunodéprimées infectées par *Cryptosporidium parvum* et *Toxoplasma gondii* avirulent. Les souris étaient infectées soit par des oocystes de *Cryptosporidium* soit par des kystes de *Toxoplasma*. L'évaluation se faisait par les taux de mortalité, les numérations parasitaires et les études par microscopie électronique. Les taux de mortalité étaient considérablement réduits dans tous les groupes traités. Une réduction considérable du nombre des oocystes cryptosporidiens dans les selles et les villosités intestinales et des kystes de *Toxoplasma* dans le cerveau des souris infectées a été observée dans tous les groupes. L'effet du médicament était plus important lorsqu'il était administré avant l'infection.

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Introduction

Cryptosporidium parvum and *Toxoplasma gondii* are opportunistic coccidial parasites that can be life-threatening in immunodeficient patients. Cryptosporidia are now recognized as common enteropathogens of both animals and humans, causing severe diarrhoea [*1*]. Toxoplasmic encephalitis is considered to be a major health problem in such patients [*2*].

More than 60 drugs have been evaluated in the treatment of cryptosporidiosis, particularly in immunocompromised patients, but none has proved to be effective in eliminating the parasite [*3*]. The administration of bovine colostrum containing *C. parvum*–specific antibodies has been shown to diminish the intensity of infection [*4*].

Toxoplasmosis requires extended treatment. Pyrimethamine and sulfonamides (mainly sulfadiazine) have been successfully used but these drugs have adverse effects in more than 40% of patients [*5*]. Recently, azithromycin, clarithromycin with or without pyrimethamine, doxycycline and atovaquone have given good results, but significant toxicities have again been observed [*6–8*].

Dehydroepiandrosterone (DHEA) is a steroid hormone produced naturally by the adrenal cortex [*9*]. Administration of exogenous DHEA has been shown to increase the life spans of animals and up-regulate their immune system [*10,11*]. DHEA is currently one of several immunomodulators undergoing clinical evaluation as a potential treatment for patients with acquired immunodeficiency syndrome (AIDS) [*12*].

These observations prompted our interest in DHEA as a therapy for the most common human coccidial infections, namely toxoplasmosis and cryptosporidiosis.

Material and methods

Parasites

C. parvum oocysts were purified from human faeces using discontinuous sucrose gradients and stored in 2.5% potassium dichromate at 4 °C. Just prior to use, oocysts were washed with RPMI 1640 (Sigma No. 7755) medium three times to remove potassium dichromate [*13*].

T. gondii cysts (avirulent strain) were used. The avirulent strain of *T. gondii* is maintained in our laboratory by passage in Swiss albino mice. Brains of infected mice were collected and homogenized in phosphate buffered saline (PBS), pH 7.2 [*14*].

Drugs

Cyclophosphamide (Endoxan) was given intraperitoneally at a dose of 70 mg/kg/mouse weekly to the end of the experiment [*15*].

DHEA (Sigma) was given subcutaneously at a dose of 120 µg/g/daily [*16*] for 3 weeks, following different schedules defined in a pilot study.

Animals

Swiss albino mice were used in the study. All of them were immunosuppressed with cyclophosphamide 2 weeks prior to the start of the study.

The mice were divided into 2 main groups: the control group and the experimental group. The control group (GI) was further subdivided into:

- GIA (drug control group): immunosuppressed mice receiving DHEA as above.
- GIB (infected control group) was further subdivided into:
  - GIB(a): mice infected with *C. parvum* at a dose of 106 oocysts in 100 µL RPMI 1640 orally [*16*].

- GIB(b): mice infected with *T. gondii* tissue cysts at a dose of 10 cysts/mouse in 100 µL of saline orally [*17*].

The experimental group (GII) was further subdivided into:

- GIIA (*C. parvum*-infected, treated mice). This group was further subdivided into:
  - GIIA(a): mice receiving DHEA 7 days prior to infection.
  - GIIA(b): mice receiving DHEA on the sixth day post-infection.
- GIIB (*T. gondii*–infected, treated group). This was further subdivided into:
  - GIIB(a): mice receiving DHEA 1 week prior to infection.
  - GIIB(b): mice receiving DHEA 6 weeks post-infection.

The mice in GIIA were killed 4 weeks post-infection and those in GIIB were killed 9 weeks post-infection.

Each experimental group had a corresponding group of 20 mice to enable the mortality rate at the end of each experiment to be calculated.

Efficacy

The efficacy of DHEA was evaluated by the mortality rate and by parasitology. For the latter, *C. parvum* oocysts were counted per high power field in the stool and per villus in the intestine. The *T. gondii* cyst count was performed after homogenization of each mouse brain in 1 mL sterile saline; cysts/100 µL were counted. In addition, ultrastructural morphology of the parasites was carried out using scanning (JEOL JSM 25 8 II) [*18*] and transmission electron microscopy (JEOL JEM 100 CX) [*19*].

Results

Mortality rates were determined in the different groups as shown in Table 1. The severity of infection in each case is given in Table 2. Prophylactic administration of the drug in cryptosporidiosis caused a reduction of 96.3% in the stool and 93.5% in the intestine count, while mice who received the drug post-infection showed 77.4% and 66.7% reductions respectively compared to the infected control. Similarly, for toxoplasmosis the reduction in brain cysts was more pronounced in the prophylactically treated group [GIIB(a)] than in the group receiving the drug post-infection [GIIB(b)] (Table 2).

Ultrastructural findings

Scanning electron microscopy (SEM) (Figures 1 and 2) demonstrated a noticeable difference between the intestinal villi of

Table 1 **Mortality rates in the different groups studied**

Group	No. of mice/group	Cryptosporidiosis		Toxoplasmosis	
		No. of deaths	Mortality rate (%)	No. of deaths	Mortality rate (%)
Drug control group (GIA)	10	0	–	1	10
Infected group (GIB)	20	9	45	6	30
Infected-treated group (GII)					
Treated before infection	20	2	10	2	10
Treated post-infection	20	4	20	3	15

Table 2 **Mean number of cryptosporidial organisms and *Toxoplasma gondii* cysts in different groups and their percentage reduction (%R)**

Group	Cryptosporidiosis				Toxoplasmosis	
	Stool oocyst/HPF		Intestinal organism/villi		Brain cysts/0.1mL	
	Mean ± *s*	%R	Mean ± *s*	%R	Mean ± *s*	%R
Infected group (GIB)	2.7 ± 1.57	–	3.1 ± 0.99	–	95.42 ± 3.57	–
Infected-treated group (GII)						
Treated before infection	0.1 ± 0.05	96.3	0.2 ± 0.57	93.5	10.79 ± 0.15	88.7
Treated post-infection	0.7 ± 0.74	77.4	0.9 ± 0.71	66.7	39.27 ± 2.43	58.8

P-value for all groups < 0.005 (significant).
HPF = high power field.
s = *standard deviation.*

the cryptosporidia-infected control group [GIB(a)] and the group of mice who received DHEA (GIIA). At the higher magnification of Figure 1, the different stages of cryptosporidia are visible, scattered between the microvilli (Figure 3). Mature schizonts are clearly visible in Figure 4.

Transmission electron microscopy (TEM) is the only method for distinguishing the stages of cryptosporidia in different groups. Figures 5 to 9 demonstrate the different stages in the infected control group [GIB(a)], namely trophozoite, immature schizont, mature schizont and microgametocyte.

However, TEM of GIIA showed an intact brush border, with parasites eradicated in many sections and scanty in others (Figure 10). The parasites detected had degenerated and were malformed, and therefore their stages could not be identified.

SEM of the *T. gondii*-infected control group [GIB(b)] showed the cyst of *T. gondii* with its homogeneous cyst wall. The wall showed minute budding, representing the bradyzoites (Figure 11). Cyst deformity was observed in mice that received DHEA (GIIB) (Figure 12). TEM of these cysts showed degeneration of many bradyzoites while others remained normal. Irregularity of the wall was also observed (Figure 13).

Discussion

C. parvum is a coccidial protozoan that causes protracted and severe diarrhoea in immunocompromised patients, especially in patients with AIDS and in malnourished children in developing countries. *T. gondii* is also considered a serious problem due to the high incidence of toxoplasmic encephalitis in immunosuppressed patients.

In this study, DHEA treatment of immunosuppressed mice infected with *C. parvum* was evaluated. The drug significantly reduced both faecal oocyst shedding and parasite colonization in ileal sections ($P < 0.005$). These findings are supported by previous results using *C. parvum* in which DHEA in immunosuppressed Syrian golden hamsters and rats [*16,20*] significantly re-

duced the infection, monitored by standard histological examination of intestine, counts of oocyst shedding and immunological assays. Similar results have been obtained in neonatal calves who received hyperimmune bovine colostrum for prophylaxis of cryptosporidiosis [*4*]. Paromomycin sulfate (Gabbroral) had a destructive effect on all stages of *Cryptosporidium*, with reduction in mucosal changes when the drug was given early in the infection but no effect when it was given later [*21,22*].

The efficacy of DHEA on cryptosporidial infection encouraged us to attempt a first trial in the management of toxoplasmosis. *T. gondii* cyst counts in the brains of mice who received the drug before and after infection were significantly reduced.

Figure 1 **SEM of intestinal villi of cryptosporidia-infected mice, showing different parasite stages (arrow) scattered between the microvilli of the brush border (× 5000).**

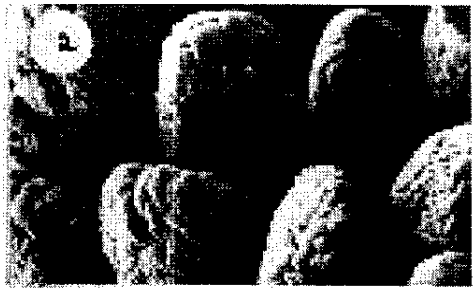

Figure 2 **SEM of intestinal villi of mice who received DHEA. The villi had normal architecture, the brush border was intact and the parasites had been eradicated in many sections (× 5000).**

Figure 3 **Higher magnification of Figure 1 showing the different sizes of different stages of *C. parvum*, each with smooth pellicle (arrow) (× 10 000).**

Figure 4 **SEM of mature schizont showing smooth transparent pellicle through which the banana-shaped merozoites can be seen lying side by side in intestinal villi of cryptosporidia-infected mice (× 13 000).**

Figure 5 **TEM of trophozite between the microvilli (mv) of cryptosporidia-infected mice. The parasite is rounded (4–6 mm) with a large nucleus (N) containing a prominent nucleolus (n) and a mesh of endoplasmic reticulum (ER). The attachment zone (AZ) is also visible (× 5000).**

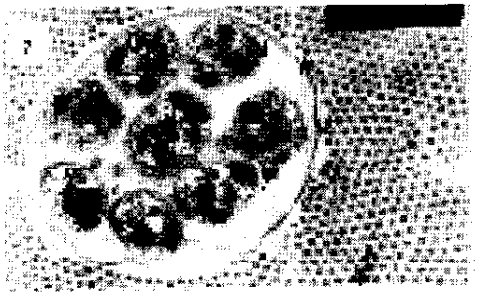

Figure 7 **TEM of mature *C. parvum* schizont of the infected control group. It has a double-walled membrane (W) and 8 well-formed mature merozoites (M). Small residual bodies (arrow) are also observed. Each merozoite is surrounded by double-walled membrane, nucleus (N) with nucleolus (n) rough endoplasmic reticulum (ER) and specific organelles [rhoptries (r) and micronemes (m)] (× 13 000).**

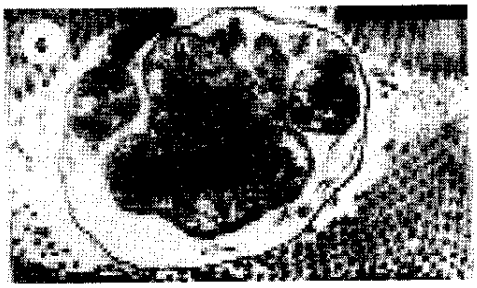

Figure 6 **TEM of an early immature *C. parvum* schizont of the infected control group. It is larger than the trophozoite and contains a number of immature merozoites (M) (× 13 000).**

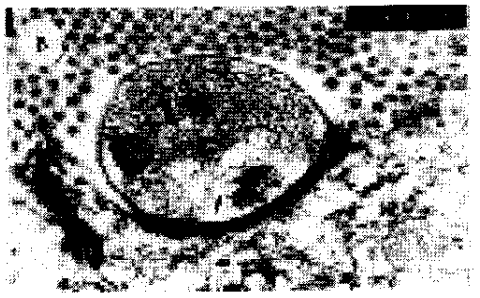

Figure 8 **TEM of microgametocytes of *C. parvum* of the infected control group. The cytoplasm is vacuolated (V) and has electron-dense granules (arrows) (× 20 000).**

Figure 9 **TEM of intestinal section of cryptosporidia-infected group, showing microgametocyte with peripheral dense compact nuclei (N) of microgametes (arrow). Immature schizont (S) is also apparent (× 10 000).**

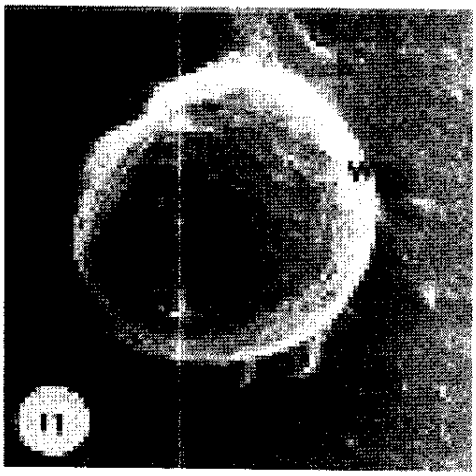

Figure 11 **SEM of *T. gondii*-infected control group showing 40–100 μm cyst, rounded with homogeneous transparent wall (W) enclosing budded bradyzoites (B) (× 2000).**

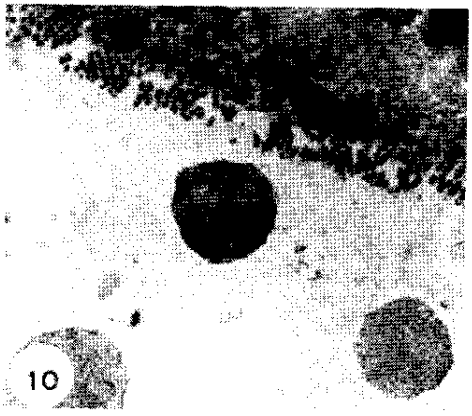

Figure 10 **TEM of intestinal section of mice which received DHEA, showing intact brush border. The parasite is malformed, opaque and shrunken with irregular outlines (arrow) (× 10 000).**

Figure 12 **SEM of *T. gondii*-infected mice which received DHEA, showing greatly disfigured cyst with crumbled surface and vesicle (V) (× 5000).**

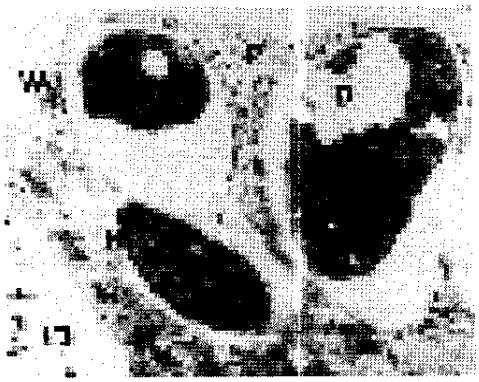

Figure 13 **TEM of *T. gondii* cyst of mice who received the drug showing degenerated bradyzoites (D) compared to normal bradyzoites (N). The wall (W) is irregular. Membranous materials (M) and dark pigmentation (P) are also seen in the spaces between the zoites (× 10 000).**

The use of clarithromycin alone in the treatment of chronically infected mice has been reported to produce a reduction of 76.6% in toxoplasma brain cyst counts, with wide variation in its effect against different strains of the parasite [*23*]. Clinical trials using the combination of sulfonamide and trimethoprim or pyrimethamine or trimetrexate–leucovorin for the treatment of refractory toxoplasmic encephalitis in the AIDS population are currently under way [*2,24*]. Unfortunately, many of these combinations have serious side-effects including allergic reactions [*2*].

In this study, the effect of DHEA on both *C. parvum* stages and *T. gondii* cysts was greater when it was given prior to infection. This could be attributed to early activation of the immune system after DHEA treatment before infection, enabling it to attack and destroy the organisms prior to invasion of host cells. When the drug was given post-infection, it was less effective in reducing *C. parvum* forms and minimizing the degree of infection in *T. gondii*–infected mice. This may be due to formation of hidden intracellular forms of *C. parvum* and to the rigid cyst wall formed around *T. gondii* organisms that escape the immunostimulatory effect of the drug.

Results of the scanning and transmission electron microscopic studies of both *C. parvum* and *T. gondii* in immunosuppressed infected treated mice paralleled the parasitic counts.

In the control immunosuppressed *C. parvum*-infected group, heavy infection by all stages was observed in all the sections. Description of the different forms was similar to previous studies [*25,26*]. In contrast, the immunosuppressed infected treated group showed eradication of the parasites in most sections and restoration of the normal architecture of the villi and the brush border. This was clearer in the group receiving early treatment. The remaining scanty parasites were malformed and hard to identify. This explains the higher percentage reduction in oocyst count in stool compared to that in the villi. Thus, DHEA in the present study had a profound distorting effect on all stages before and late in *C. parvum* infection, and is therefore superior to other drugs both as a treatment and as prophylaxis.

SEM studies of *T. gondii*–immunosuppressed treated mice revealed deformation of the cyst outlines with indentation and vesicle formation. TEM showed progressive degeneration of most zoites with disruption of the cyst wall. In the spaces between the zoites membranous material and dark bodies could be seen.

As far as we know, there are no other reports of electron microscopy of these parasites following treatment with DHEA.

However, Khalifa and Sharaf El-Din [*22*] reported similar SEM results using paromomycin sulfate in experimental cryptosporidiosis. Sarciron et al. [*27*] used the antiviral agent 2,3-dideoxyinosine against the avirulent DUR strain of *T. gondii in vitro*, and by studying its effect on the ultrastructural level found that it had a striking activity as an antitoxoplasma drug at low doses. Lindsay et al. [*28*] tested the anticoccidial agent diclazuril on the *in vitro* development of 3 strains of *T. gondii*. Using TEM, they obtained results similar to ours when the drug was given at a dose of 1 μg/mL. However, it needed 2 days to show an effect, formation of tissue cysts was not prevented and bradyzoites released from the cysts were resistant to the treatment. This drug also significantly reduced the mortality rate in cryptosporidial- and toxoplasma-infected groups of mice.

Our findings suggest the effect of DHEA could be attributed to its immunomodulatory effect. Interestingly, Casson et al. [*29*] demonstrated that oral DHEA modulates immune function in postmenopausal women. DHEA is one of 30 new agents and agent combinations currently being evaluated in cancer chemoprevention [*30*]. Treatment with DHEA alone in the immunocompetent host produces a measurable increase in B and T cell blastogenesis, serum immunoglobulin (IgG) levels and IgG production *in vitro*. This indicates that DHEA stimulates the immune response in the absence of immunosuppression, and could be of help in the treatment of opportunistic infections. Moreover, it has been reported that DHEA supplementation decreased CD4+T cells and increased CD8+/CD56+ [natural killer (NK)] cells, with an increase in NK cytotoxicity. Published evidence suggests that NK cells have lytic activity against intracellular pathogens and defects in NK cell activity have been described in AIDS patients [*20,31*]. Recently, DHEA was shown to induce a significant up-regulation of interleukin-2 (IL-2) production by normal T cells, suggesting that administration of exogenous DHEA or IL-2 to mice with autoimmune disease dramatically reverses the clinical manifestations [*32*]. In terms of its effect on cryptosporidiosis, Daynes et al. [*33*] demonstrated that DHEA selectively enhanced the production of both IL-2 and interferon (IFN) by activated helper T cells and so might help both the cellular and the humoral arms of the immune system. Rasmussen et al. [*20*] suggested that the effect of DHEA on reducing cryptosporidiosis may be due to the increased production of both IL-2 and IFN required for clonal proliferation of antigen-activated T cells. Our results in toxoplasmosis are supported by the work of El-Nassery et al. [*34*], who reported that IL-2 given to Swiss albino mice had a significant destructive and deforming activity on *T. gondii* tachyzoites, as IL-2 stimulates NK cell activity as well as macrophage cytotoxicity. This could explain the significant reduction in *T. gondii* cyst count and control of spread of infection in the treated group that we observed. We speculate that treatment with DHEA in both *C. parvum* and *T. gondii* infection reduced the colonization (in *C. parvum* infection) and spread of infection (in *T. gondii* infection) in the immunosuppressed experimental mice as a result of the direct stimulatory effect of DHEA on the immune system.

In conclusion, DHEA can successfully control and treat cryptosporidiosis and reactivated toxoplasmosis as well as combined coccidial infection in immunocompromised patients. It is a promising and effective new agent lacking many of the serious side-effects of other anticoccidial therapies.

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