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Research Article

Sex Steroids Effects on the Molting Process of the Helminth Human Parasite *Trichinella spiralis*

Romel Hernández-Bello,[1] Ricardo Ramirez-Nieto,[1] Saé Muñiz-Hernández,[2] Karen Nava-Castro,[3,4] Lenin Pavón,[5] Ana Gabriela Sánchez-Acosta,[1] and Jorge Morales-Montor[1]

[1] *Departamento de Inmunología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, 04510 México, DF, Mexico*
[2] *Subdirección de Investigación Básica, Instituto Nacional de Cancerología, Secretaría de Salud, 14080 México, DF, Mexico*
[3] *Departamento de Inmunología e Infectología Pediátrica, Instituto Nacional de Pediatría, Secretaría de Salud, 11000 México, DF, Mexico*
[4] *Departamento de Biología, Facultad de Química, Universidad Nacional Autónoma de México, 04510, Mexico*
[5] *Departamento de Psicoinmunología, Instituto Nacional de Psiquiatría "Ramón de la Fuente", 14370 México, DF, Mexico*

Correspondence should be addressed to Jorge Morales-Montor, jmontor66@biomedicas.unam.mx

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We evaluated the *in vitro* effects of estradiol, progesterone, and testosterone on the molting process, which is the initial and crucial step in the development of the muscular larvae (ML or L1) to adult worm. Testosterone had no significative effect on the molting rate of the parasite, however, progesterone decreased the molting rate about a 50% in a concentration- and time-independent pattern, while estradiol had a slight effect (10%). The gene expression of caveolin-1, a specific gene used as a marker of parasite development, showed that progesterone and estradiol downregulated its expression, while protein expression was unaffected. By using flow citometry, a possible protein that is recognized by a commercial antiprogesterone receptor antibody was detected. These findings may have strong implications in the host-parasite coevolution, in the sex-associated susceptibility to this infection and could point out to possibilities to use antihormones to inhibit parasite development.

1. Introduction

The sex steroids hormones, 17β-estradiol (E_2), progesterone (P_4), and testosterone (T_4), act upon the reproductive system of mammalians by binding to specific sex steroid receptors, which determine changes in reproductive physiology and behaviour [1, 2]. Recently, it has been shown that sex steroids participate not only in reproductive physiology, but also in a number of different functions, which include immune modulation, brain activity, bone metabolism, and lung and heart physiology. Moreover, also strong direct effects of sex steroids on parasites to modulate different parasite functions have been reported [3–7].

Recent information reveals that sex hormones can affect the course of worm infection [8–12], as in the case of the cestodes *Taenia crassiceps* and *Taenia solium* [13–15]. In line with this statement, it is known that the frequency of *T. solium* pig cysticercosis is increased during pregnancy, when there is a significant increase in progesterone levels [13, 16]. It has also been demonstrated that the castration in naturally infected male boars induces an increase in the prevalence of cysticercosis, which highlights the possible role of host androgens to restrict parasite establishment and estrogens to facilitate it [13]. Furthermore, another helminth, *T. crassiceps* (a close relative of *T. solium*) has shown to be affected by *in vitro* sex steroid treatment. Specifically, 17β-estradiol increases the reproduction of *T. crassiceps* parasites, while testosterone or dihydrotestosterone decreases it [17]. *In vivo,* when castrated mice are treated with 17β-estradiol, the number of parasites as well as their infective capacity

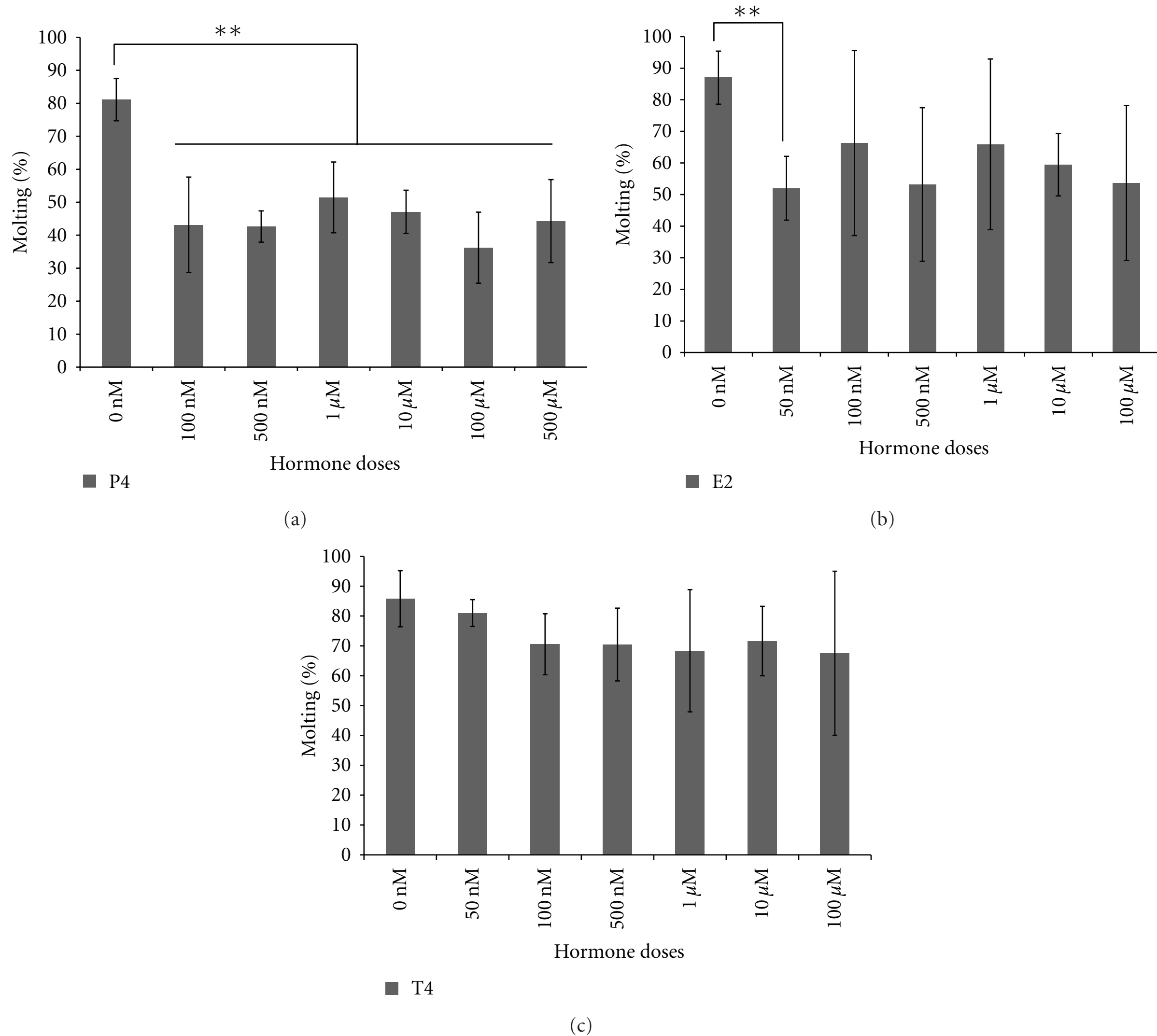


Figure 1: Dose-dependent curves of sex steroid hormones on *in vitro* molting of muscle larve of *T. spiralis*. One hundred muscle larve (ML) of *T. spiralis* were incubated for 36 hours with different concentrations of progesterone (P4), estradiol (E2), or testosterone (T4). Progesterone inhibited the molting rate of the muscle larva of *T. spiralis* in a concentration-independent pattern (a). Estradiol inhibit only at 50 nM concentration (b), while testosterone has no any effect on molting (c). Each point represents the mean (SD) of quintuplicate determinations of the number of larvae in molting process.

increases up to 200% [7, 18] meanwhile progesterone has the opposite effect in castrated mice of both sexes: a decrease in the parasite loads of almost 100% [19].

T. spiralis is an intracellular parasitic nematode of mammalian striated muscles. The life cycle of this parasite is completed within a single host and the parasite resides in two distinct intracellular habitats. The infective stage of *T. spiralis* is part of a nurse cell-larva complex found in striated muscle of prey eaten by carnivores. Digestive enzymes in the stomach release the larva from the muscle tissue and the parasitic L1 migrate to small intestinal sites at the base of villi where they reside in a syncytium of epithelial cells.

Consistent with the level of coevolution evident from parasite adaptation to the host is the assumption that *T. spiralis* can exploit the hormonal microenvironments within the host [8]. This suggests a system of transregulation (term coined by us) in which the parasite exploits host hormones and growth factors to facilitate infection and potentially increase growth and reproduction rates; this process has been described in at least eight parasitic infections that are caused by both protozoan and metazoans [10]. Furthermore, endocrine factors, related to sex and age, are well recognized as modulators of the immune response [11, 12] and by having a direct effect over the parasite. Thus, sex steroid hormones play key roles in the susceptibility to trichinellosis at two levels: (a) protective immune response or (b) direct effect on parasite development [11, 15]. Steroid hormone effects are not only restricted to cestode parasites, but also to nematodes such as *Ancyclostoma dudodenale*, whose number of larval and adult stages is increased by sex steroid hormones in several organs of mice [20]. Moreover, adult and muscle larvae of *T. spiralis* are increased in ovarectomized female

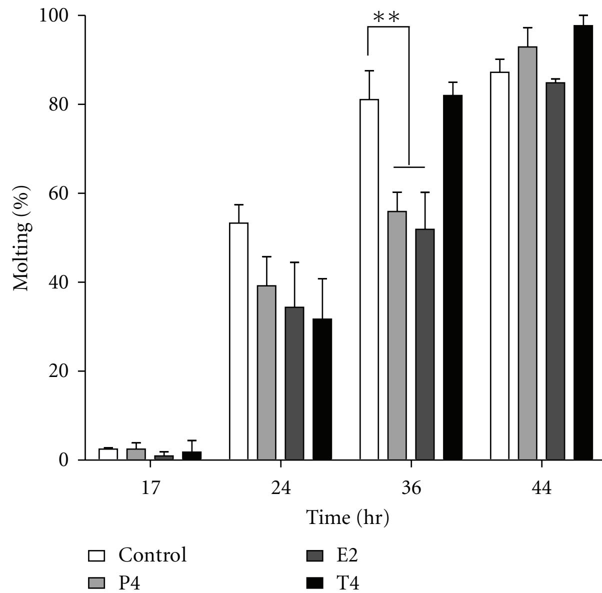


Figure 2: Time curves of *T. spiralis* molting process after exposure to progesterone (P4), estradiol (E2), and/or testosterone (T4). The parasites were cultured for 44 hours. Progesterone and estradiol had 35–50% of inhibitory effect on molting rate compared with control and testosterone. The maximum inhibitory effect was at 36 hr of culture. Each point represents the mean (SD) of 5 assays counting the number of molting larvae and the mean was obtained. $^{**}P < 0.05$. The hormone concentration was as follows: P4, 100 mM; E2, 50 nM, and T4, 50 nM.

rats [10], suggesting that estrogens and progesterone are restrictive factors for parasite establishment, while androgens should play a permissive role to the infection. In the case of the nematode *T. spiralis,* males are generally more susceptible than females to the infection, since in mice the males present more parasite burden at both intestinal and muscle level than females do [21–23]. This finding may represent an interesting approach in trichinellosis by *T. spiralis*, because if we know that sex steroids can specifically down-regulate genes involved in the fecundity and oviposition of the parasite, we can propose the use of sex steroid analogous to modulate this effect. Taking into consideration this information, the aim of the present study was to explore the role of sex steroids on *T. spiralis* development, evaluating its *in vitro* effects on the molting process, which is the key in the maintenance of the infectious cycle in the host. The *in vitro* effect of progesterone, estradiol, and testosterone on *T. spiralis* was studied through pharmacological and molecular (RT-PCR, immunohistochemistry and flow cytometry) approaches, in order to figure out the mechanism of sex steroids actions in the parasite.

2. Materials and Methods

2.1. Obtention of Parasites. T. spiralis was maintained in the laboratory by serial passage infections in Wistar rats. The infective-stage ML were recovered as described in [24]. Briefly, the carcass of experimentally infected mice at 30 days of infection was digested by a standard pepsin-hydrochloric acid digestion method to obtain LM stage.

2.2. Sex Steroids Dose-Response Time Curves. Culture grade estradiol, progesterone, and testosterone were obtained from Sigma (Sigma-Aldrich, USA). For *in vitro* tests, estradiol and progesterone water soluble (Sigma-Aldrich, USA) were dissolved in RPMI 1640 free serum culture medium (Gibco) at desired stock concentration and sterilized by passage through a 0.2 mm millipore filter. Testosterone was dissolved in absolute ethanol to the desired stock concentration and sterilized by passage through a 0.2 mm millipore filter. For cultures, the experimental design was as follows: 100 muscular larvae were cultured in 24-well plates and 6 wells per condition were used. Groups designed were as follows: control (only RPMI); control vehicle (EtOH-RPMI); estradiol; progesterone; testosterone. For time-response curves, parasites from all treatments were cultured during 48 hr, with medium change at 24 hr, using optimal physiological concentration for estradiol (50 nM), progesterone (100 nM), and testosterone (50 nM). Culture wells contained 2 mL of RPMI medium and were incubated at 37°C and 5% CO_2. Steroids were prepared to add 100 μL to 5 mL of the medium in each well to final concentration. From concentration-response curves of each steroid, we selected physiological concentration for estradiol, progesterone, and testosterone, as well as, increased concentration of each in pharmacological concentration (above 10 μM concentration). Survival and molting were determined under light microscopy using Axiovert Ziess Microscope and 25x Neo Plan objective.

2.3. Morphologic Analysis of T. spiralis Treated with Sex Steroids. T. spiralis ML cultured with or without hormones were observed at different hours under light microscopy using Axiovert Ziess Microscope and 25x Neo Plan objective. The microphotographies generated were modified and contrasted using a software image (Adobe Photoshop CS3, US).

2.4. RNA Extraction of Cultured Parasites in Presence of Sex Steroids. Total RNA was isolated from *T. spiralis* of each *in vitro* treatment (positive expression control) using Trizol reagent (Invitrogen, Carlsbad, Calif.). In brief, parasites were homogenized in Trizol reagent (1 mL/0.1 g tissue), and 0.2 mL of chloroform was added per mL of Trizol. The aqueous phase was recovered after 10 min of centrifugation at 14,000 g. RNA was precipitated with isopropyl alcohol, washed with 75% ethanol, and redissolved in RNAse-free water. Total RNA concentration was determined by absorbance at 260 nm and its purity was verified after electrophoresis on 1.0% denaturing agarose gel in presence of 2.2 M formaldehyde. Total RNA was stored at −70°C

2.5. Immunohistochemistry of Caveolin-1 (CAV-1) in the Parasite. T. spiralis ML obtained at 36 hr of culture with or without the sex steroids were embedded and frozen in tissue freezing medium (Triangle Biomedical Science, USA) at −70°C. Four-micrometre-thick sections of parasites were fixed with 4% paraformaldehyde for 30 min, permeabilised

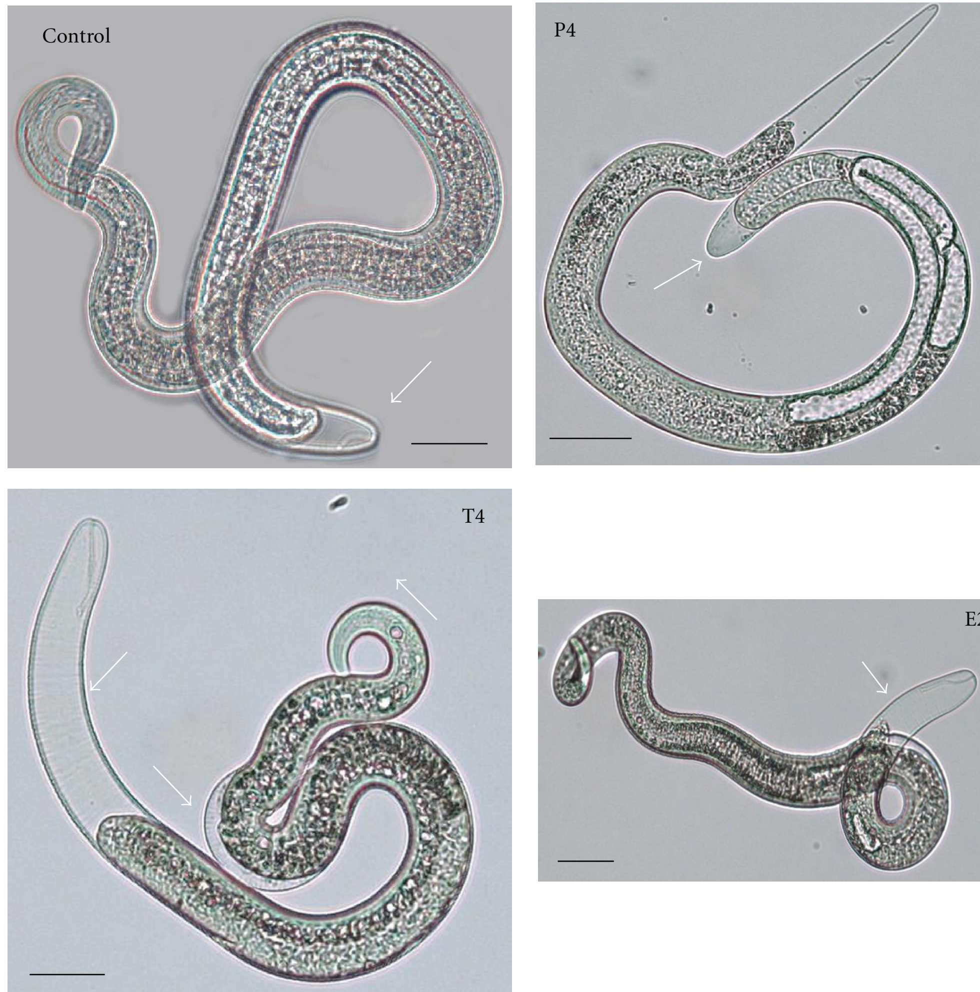


Figure 3: Micrographies of ML parasites cultures in presence of sexual steroids. Parasites cultured at 36 hr with progesterone (P4, 100 mM), estradiol (E2, 50 nM), or testosterone (T4, 50 nM) or in absence of sex steroid (control) were observed in Axiovert microscopy using 25x objective. In all cases, the old cuticle starts to detach (clear zone) at the apical or basal of the parasite (arrows).

in 1% PBS-SDS and blocked with RPMI medium containing 0.5% BSA and 5% FBS, as described in [25]. Cross-sections were incubated with the previously obtained polyclonal anti-Ts-Cav-1 antibody and then incubated with the secondary antibody, fluorescein-isothiocyanate-(FITC-) conjugated goat anti-mouse antibody (Sigma-Aldrich) [25]. Control experiments were performed by incubation secondary antibody. The background fluorescence in samples was reduced by contrast with 0.025% Evans Blue. Samples were analysed in a Leica TCSSP5 confocal laser-scanning microscope (Leica Microsystems, Germany). The images were constructed using Leica Confocal software.

2.6. Flow Cytometry to Detect Progesterone (PR) Receptor in Parasites Cells. *T. spiralis* cells were extracted by tissue disruption using a micropestle (Eppendorf, USA) until no more clumps were visible. Cells were centrifuged at 300 g for 5 min and recovered in PBS. Cells were stained with the following antibodies for 10 min at 4°C: anti-mouse CD3-FITC, and anti-mouse CD19-PE, and anti-mouse Mac-1 (all from BD Biosciences). For intracellular staining, cells were initially fixed with 2% paraformaldehyde solution for 10 min at 37°C, permeabilized with methanol 100%, washed twice with 500 μL of staining buffer (PBS pH. 7.4, 2% Fetal Bovine Serum, 0.02% NaN_2), and stained with anti-caveolin-1 and anti-PR (Santa Cruz Biotechnology, USA). For primary antibodies recognition, cells were stained with FITC coupled anti-mouse IgG and PE-coupled anti-rabbit IgG for 10 minutes at room temperature, washed, and stored until the analysis is protected from light. Ovary-derived cells were stained with anti-PR as described for *T. spiralis* cells. All samples were analyzed by flow cytometry using an FACS Calibur (BD, Biosciences, USA) and data analyzed using the FlowJo software.

2.7. Experimental Design and Statistical Analysis. Hormone dose-response time curves were estimated in 4 independent experiments. The response variable used in statistical analyses was the total number of the molted parasites that showed motility in all wells of each hormone concentration

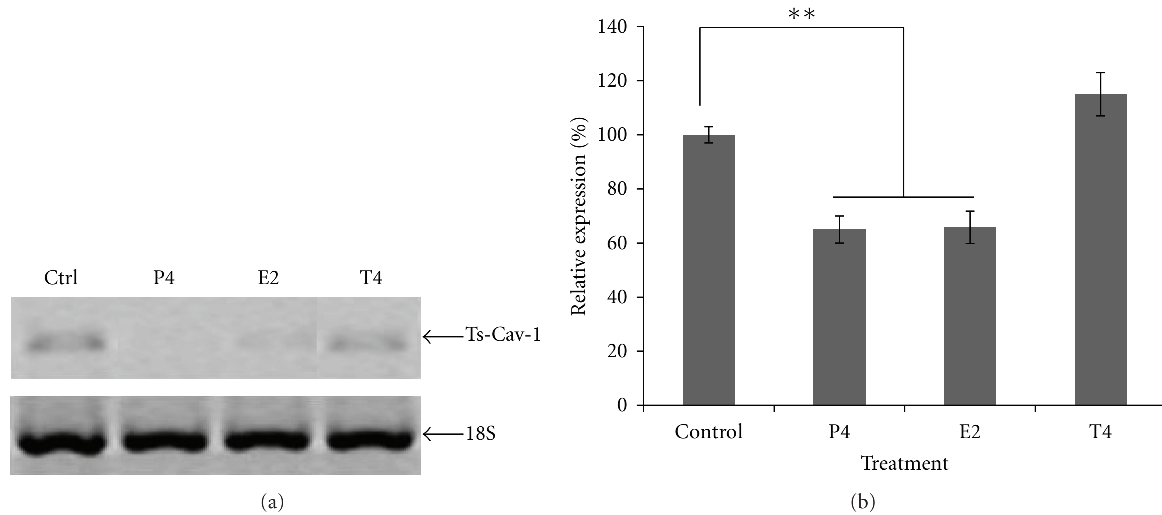


Figure 4: Ts-Cav-1 gene expression in *T. spiralis* ML cultured in presence of sex steroids. A single band of 306 bp, corresponding to the caveolin-1 of *T. spiralis,* was detected in all parasites cultured at 36 hr (a). Progesterone and estradiol reduced the expression level of this gene at least 40%, while testosterone has no effect on the expression of this gene (b). Densitometric analysis is shown (b). 18 S was used as constitutive expression gene. Data are represented as mean ± SD. $^{**}P < 0.05$.

and time of exposure, for every experiment. Hormones, their concentrations and times of exposure were the independent variables. The data for the 4 replicates of each treatment were expressed as an average. Data were analyzed using one-way variance analysis (ANOVA). If ANOVA showed significant differences among treatments, a Tukey Test was applied for test significance. Differences were considered significant when $P < 0.05$.

2.8. Ethics Statement. Animal care and experimentation practices at the Instituto de Investigaciones Biomédicas are frequently evaluated by the Institute Animal Care and Use Committee, and by governmental (official Mexican regulations (NOM-062-ZOO-1999)), in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health of the USA, to ensure compliance with established international regulations and guidelines. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Instituto de Investigaciones Biomédicas. Rats were sacrificed to obtain the L1 using sevorane as anesthesia, and all efforts were made to minimize suffering.

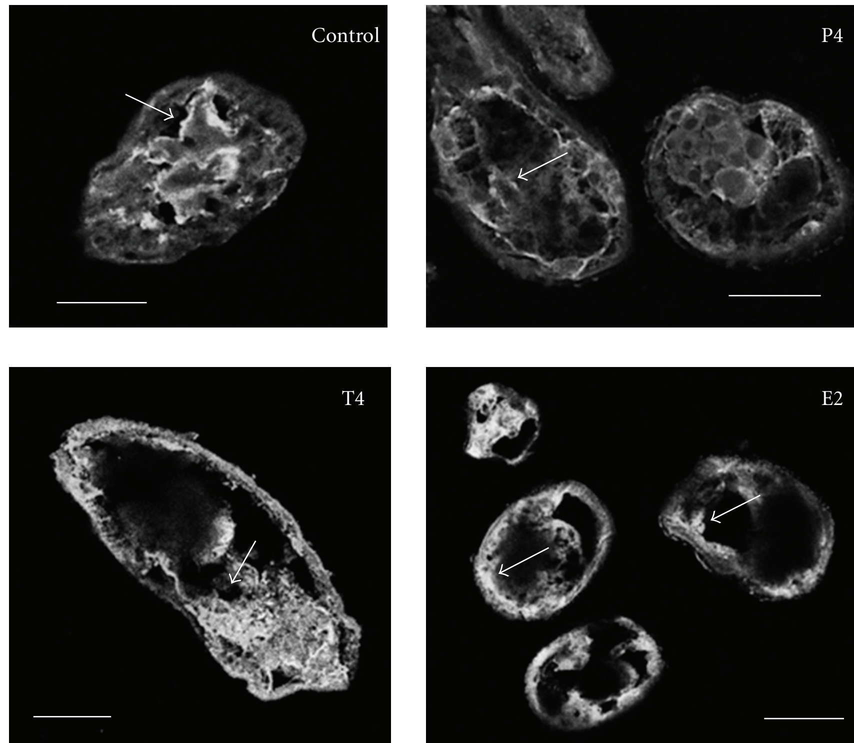
3. Results

When *T. spiralis* parasites were *in vitro* exposed to progesterone, a decrease in the molting process rate about 35–50% was observed in all treated parasites compared to control groups (Figure 1(a)). However, this molting-inhibiting effect mediated by progesterone was independent of the tested concentrations (Figure 1(a)). For estradiol, an inhibitory effect was observed for the lowest concentration, but just an inhibitory tendency was observed in all cases independent of the culture concentration tested, without any significant differences (Figure 1(b)). In the case of testosterone, there was no apparent effect on the molting of *T. spiralis in vitro* (Figure 1(c)).

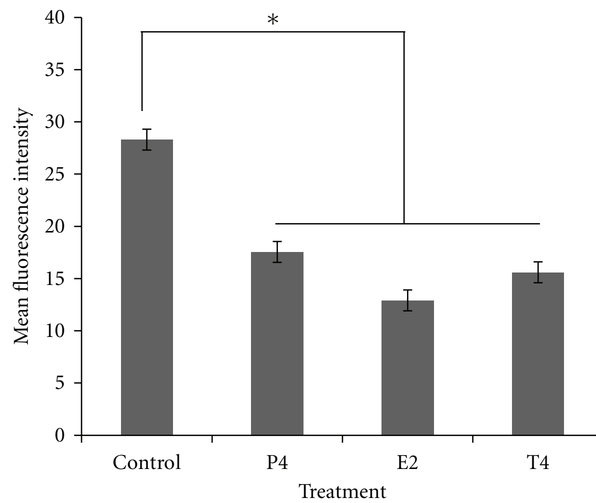
Concomitantly, the molting-inhibiting effect of progesterone (100 nM) and estradiol (50 nM) was maintained through all 40 hours of *in vitro* culture, reaching its highest response on 36 h in culture, in relation to untreated parasites (Figure 2). Consistently, when parasites were exposed to testosterone (50 nM), no differences on the percent of molting were observed neither during the first hours of culture nor at the end of the process (Figure 2).

It is important to mention that viability of the molted parasites was verified daily by means of worm motility in the culture plate, which was constant through all days of *in vitro* culture. Injured parasites were recognized by a progressive internal disorganization: development of clear areas inside of the parasite and increasing number of internal vesicules (Figure 3). Differentiated worms in absence of hormones had a normal development, reaching their tipical shape characteristic of adult at 40 hours in culture and have a normal internal organization and the molting process is ongoing well. Once again, in the presence of estradiol and progesterone, the molting rate is decreased, while in the testosterone-treated parasites, the molt rate is slightly accelerated (Figure 3).

In order to look for molecular effects of sex steroids, we sort out the expression of caveolin-1 (Cav-1), which is a marker protein for oocyte development in the female worm of *T. spiralis*. A single band corresponding to the expected molecular weight of the amplified fragment of Cav-1 (approximately 306 bp) was detected from *T. spiralis* control and treated with sex steroids (Figure 4). Relative expression of the mRNA of the Cav-1 gene in the parasite revealed that estradiol and progesterone decrease the expression by 40%, while treatment with testosterone increased the same by 10% (Figure 4).



(a)



(b)

Figure 5: Indirect immunofluorescence of Ts-Cav-1 protein expression in muscle larvae (ML) cultured. Four-micrometre sections from parasites cultured without or with sex steroids (P4, E2 or T4) were treated with anti-Ts-Cav-1 antibody and fluorescein isothiocyanate-conjugated goat anti-mouse antibody and observed under confocal laser microscopy. In all cases of parasite treated there was no changes on Ts-Cav-1 protein expression. Bars = 25 μm.

Interestingly, when we look for the expression pattern of Cav-1 by immunohistochemistry, there were no apparent differences between the untreated parasites and the ones exposed to sex steroids (Figure 5(a)). When we quantified this pattern of expression, this pattern was confirmed, since quantification of cells expressing Cav-1 was no different when we compared between treatments (Figure 5(b)).

In order to determine if the effect of progesterone was mediated by a putative progesterone receptor in the parasite, we perform flow cytometry using commercial anti-PR antibodies. As shown in Figure 6(a) (upper row), *T. spiralis* cells do not express immune cell markers such as CD3, CD19, or Mac-1, which are typically present in some types of mammalian leukocytes, which suggest that there

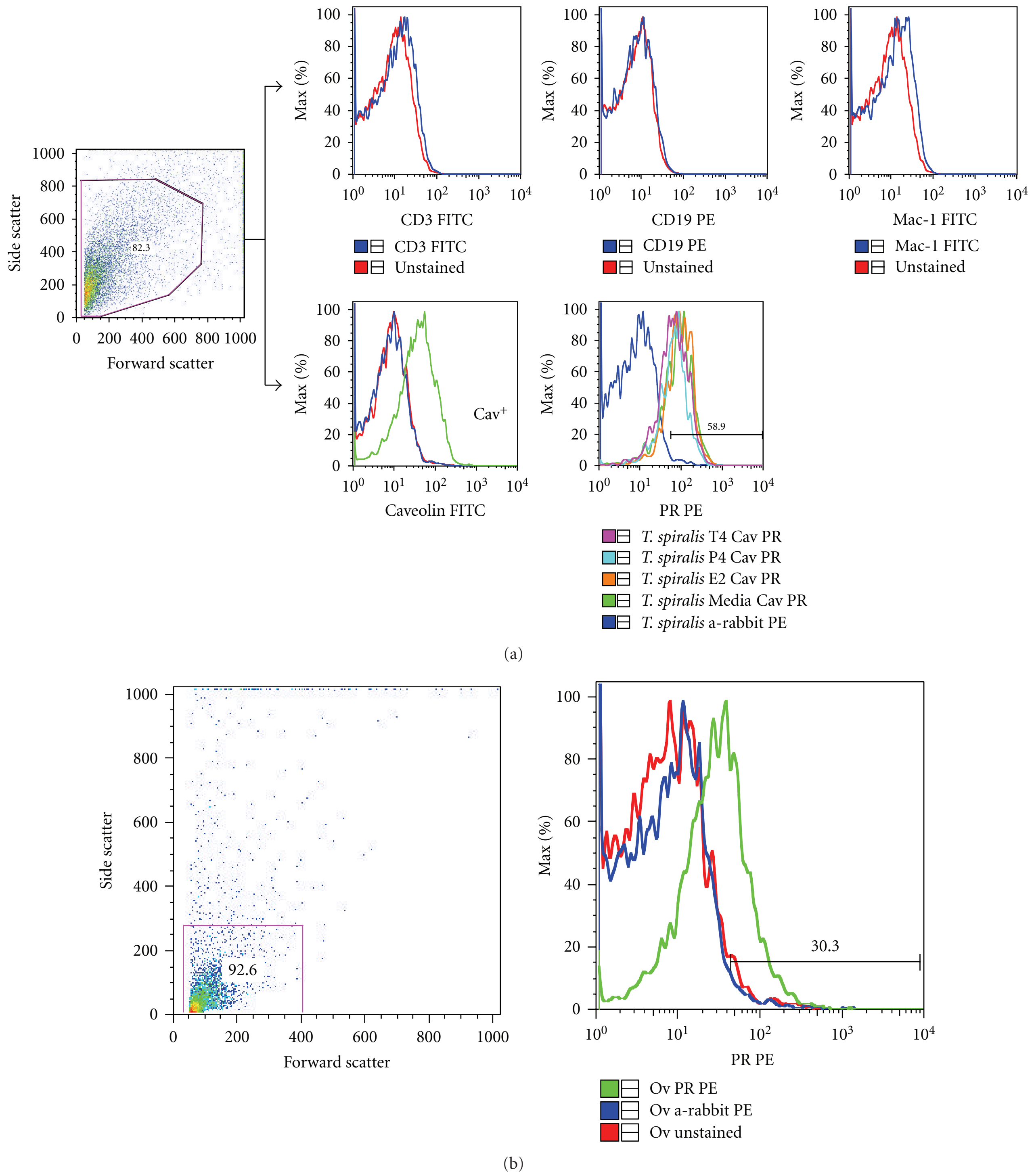


Figure 6: Progesterone receptor (PR) and host markers expression on *T. spiralis* cells. (a) *T. spiralis* cells were stained with anti-mCD3, anti-mCD19, and anti-mMac-1 (upper row); cultured in presence of media (green line), E2 (orange line), P4 (blue line), and T4 (pink line) and stained with anti-Cav-1 of *T. spiralis* and anti-PR specific antibodies (middle row). (b) Ovary-derived cells were stained with anti-PR antibody as a positive control for PR.

is no contamination with host cells. We then analyzed the expression of the classic progesterone receptor (PR) on parasitic cells that expressed caveolin-1 (a protein cloned, sequenced, and expressed exclusively by this parasite), in response to the steroid hormones estradiol, progesterone, and testosterone. As shown in Figure 6(a) (middle row), cav$^+$ cells showed a specific binding of anti-PR antibody, which suggested the presence of a PR-like receptor of parasitic

origin. PR expression was also detected in ovary, as an expression control tissue (Figure 6(b)).

4. Discussion

Estrogens and androgens play an important role at different system levels for maintenance of homeostasis in vertebrates. They are implicated not only on the reproductive behavior and physiology, but also act as modulators of immune system, brain activity, and lung and heart physiology. In the last years, increasing information reveals that sex hormones can affect the course of parasite infection [8, 9, 11, 12] by modulating the Th1 or Th2 response to lead a susceptibility/resistance to infections. In that way, sex differences on parasitemia can reflect the suppressive effects of testosterone and the increasing effects of estrogens in the immune system [26], principally on Th2 immune responses in females leading to a higher production of interleukins (i.e., IL-4, -5, -6 and -10) [27]. Therefore, males of several species are more susceptible to infections caused by different parasites as well as the prevalence and intensity of parasitic infection than females do [28, 29]. For example, male mice are less resistant to protozoan like *Plasmodium berghei* [30] or *Trypanosoma cruzi* [31] or nematodes like *Strongyloides sp*. [32] than females, while the administration of potent estrogenic compounds as estradiol increased the resistence to the parasite [31–33]. Otherwise, progesterone protects mice from *T. crassiceps* infections [19].

Particularly on *T. spiralis* infections, it has been described a resistance/susceptibility associated to gender's host; in which males are more susceptible than females [22, 23]. Interestingly, in pregnant rats, where progesterone levels is increased, the resistance to *T. spiralis* infections throught parasite loads in muscle are increased compared to parasite loads in muscle observed in virgin rats. This effect was mediated by the ability of sera to mediate death in newborn larvae (NBL) of *T. spiralis* in antibody-dependent cell cytotoxicity assays [34]. Also, progesterone can activate effector peritoneal cells to eliminate NBL in a rapid and antibody-independent manner [35]. However, the intervention of the host's immune response in dealing with the parasite and the possibility of additional direct effects of sexual steroid hormones on the parasite's physiology should not be hastily discarded. Here we describe the effects of progesterone, estradiol, and testosterone upon the molting process of *T. spiralis* larvae. First of all, it was clear that progesterone has a direct inhibitory effect on the percent of worms that are going through the molting process *in vitro*. In fact, progesterone exerts a marked molting-inhibitory effect in a concentration-independent pattern, maintained entire time in culture, making that all parasites differentiate at 36 hours of *in vitro* culture.

T. spiralis parasites not only showed molting, but they also presented a constant motility in the culture plate, which suggests that neither estradiol nor progesterone affected parasite viability and therefore they were alive during the culture process. Moreover, progesterone induced a decrease in the rate of the molting process of the worm in the cultured parasites, with respect to untreated parasites. Estradiol and progesterone inhibited the expression of Ts-Cav-1 in the LM, one gene that is implicated in the maturing and development of the NBL [25]. This observation result is important because it suggests that the LM of *T. spiralis* could modify the expression of specific genes through steroid receptors similar to those found in other invertebrates [36, 37]. However, no differences on expression of Ts-Cav-1 protein were observed. One explanation is because the Ts-Cav-1 protein is a molecule that is accumulated in the plasma membrane of the parasite's cells, principally oocytes. We thought that this protein generated in all treatments during the first hours of being cultured, was cumulated in the membrane, and for 36 hr of culture, we do not see any difference in the accumulated protein, although existed a down-regulation in the gene expression at this time on estradiol or progesterone cultures existed. Interestingly, neither percent molting nor molting rate depends on testosterone concentrations. These findings, could explain in part the decreased parasite loads observed in females on infection with *T. spiralis* and even in pregnant females: sex steroids present in the pregnant female environment avoid the normal rate of molting process on the LM to adult female which directly impacts in the potential adult female that releases NBL. Our results contribute to the knowledge of the mechanisms by which the host microenvironment affects the parasite and parasite protein regulation and expression. As it can be seen, direct effects of sex steroids upon helminth parasites (cestodes, nematodes, and trematodes) are not unusual. In fact, previous results suggest that these pathogens are not only directly affected by hormones, but they have also developed several strategies to exploit the host's endocrine microenvironment [4, 38], which include degradation of host proteins as an alternative source of aminoacids [39], development of parasitic-sex steroid receptors [40, 41], and cross-activation of signal transduction pathways [42, 43].

5. Conclusions

Here, we describe different effects of sex steroids that probably have different action mechanisms directly upon *T. spiralis*. Progesterone effects could explain, at least partially, the low parasite burdens observed on experimental trichinellosis infection in pregnant and in nonpregnant rats [34]. To our knowledge, this is the first report where antitrichinella effects are described for sex steroids and open a promissory field in the design of new strategies that include the antihormone therapy in the control of trichinellosis caused by *T. spiralis*.

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