TRABALHO FINAL

MESTRADO INTEGRADO EM MEDICINA

Instituto de Histologia e Biologia do Desenvolvimento

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Orientada por:

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Abstract

Trypanosoma brucei is the causative agent of Human African trypanosomiasis, also known as sleeping sickness. Disease progression usually begins with a haemo-lympathic phase, followed by parasite invasion of the central nervous system. Currently no vaccines are available and a limited range of drugs exists to treat this disease, many of them associated with high toxicity. Recent studies have revealed the presence of trypanosomes in the male reproductive system. When considering trypanocidal drug development reproductive organs are of interest, since parasites that infiltrate and persist in the male gonads may be protected from drugs by the blood-testis barrier. Since there is epidemiological evidence in humans for the sexual transmission of *T. brucei*, understanding if and how trypanosomes are distributed in the male reproductive system may also help identifying the pathways underlying sexual transmission of the disease. Here we characterized the infection in the mouse male reproductive system using an animal model and showed, through histological analysis, parasites and inflammatory cells infiltrating the male reproductive system later in the infection, especially in the epididymis. Parasite density, determined by quantitative PCR of genomic DNA, confirmed that parasite load increased overtime. Transmission electron microscopy showed that even when found in higher numbers, trypanosomes in the epididymis had severe morphological changes consistent with cell death, possibly due to the immune response seen later on. Overall, we propose that the inflammatory cell infiltration may compromise drug diffusion, enabling reproductive organs to act as a reservoir for parasites. Also, parasite distribution in epididymis may enable sexual transmission. The identification of this reservoir opens new pathways to future research concerning parasite tropism and development of new and more efficient drugs.

Keywords: Human African trypanosomiasis, Male reproductive system, Sexual transmission, Animal Models

Sumário

Trypanosoma brucei é o microorganismo responsável pela tripanossomíase africana humana, também conhecida como doença do sono. A progressão da doença geralmente começa com uma fase hemolinfática, seguida pela invasão parasitária do sistema nervoso central. Actualmente não existem vacinas disponíveis e existe um conjunto limitado de fármacos para tratar esta doença, muitos deles associados a uma elevada toxicidade. Estudos recentes revelaram a presença de tripanossomas no sistema reprodutor masculino. Ao considerar o desenvolvimento de fármacos anti-tripanossomíase, os órgãos reprodutores são de interesse, uma vez que os parasitas que se infiltram e persistem nas gónadas masculinas poderão estar protegidos dos fármacos pela barreira hemato-testicular. Uma vez que há evidências epidemiológicas da transmissão sexual de *T. brucei* em seres humanos, perceber como os tripanosomas estão distribuídos no sistema reprodutor masculino também pode ajudar a identificar os caminhos subjacentes à transmissão sexual da doença. Neste projecto caracterizámos a infecção no sistema reprodutor masculino de ratinho utilizando um modelo animal e, através de análises histológicas, identificámos a infiltração do sistema reprodutor masculino por parasitas e células inflamatórias infiltrados numa fase tardia da infecção, especialmente no epidídimo. A quantificação de parasitas, determinada por PCR quantitativo de DNA genómico, confirmou que a carga parasitária aumentou ao longo do tempo. A microscopia eletrónica de transmissão mostrou que, mesmo quando encontrados em maior quantidade, os tripanosomas no epidídimo apresentaram acentuadas alterações morfológicas consistentes com morte celular, possivelmente devido à resposta imune observada numa fase tardia. Em suma, propomos que a infiltração de células inflamatórias poderá comprometer a difusão de fármacos, permitindo que os órgãos reprodutivos atuem como um reservatório de parasitas. Além disso, a distribuição parasitária no epidídimo poderá permitir a transmissão sexual. A identificação deste reservatório abre novos caminhos para pesquisas futuras sobre o tropismo parasitário e o desenvolvimento de novas drogas mais eficazes.

Palavras-chave: Tripanossomíase Humana Africana, Sistema reprodutor masculino, Transmissão sexual, Modelos animais

Resumo

A Tripanossomíase Humana Africana, também conhecida como doença do sono, é causada pelo parasita *Trypanosoma brucei gambiense* na África Ocidental e Central *e T. b. Rhodesiense* na África Oriental. *T. b. gambiense* é responsável por cerca de 97% dos casos em seres humanos, enquanto *T. b. rhodesiense* é mais raro, mas provoca uma doença mais grave. Esta doença apresenta duas fases: a fase primária ou hemolinfática e a fase secundária ou meningoencefálica. A primeira é caracterizada pela multiplicação dos parasitas na corrente sanguínea e nos espaços intersticiais de diversos órgãos. Com a progressão da doença, inicia-se a segunda fase, na qual os parasitas invadem o sistema nervoso central, penetrando a barreira hemato-encefálica. Após tratamento a taxa de recidiva pode chegar até 30% em alguns focos da doença por *T. gambiense*. Estas recidivas foram atribuídas ao facto dos parasitas presentes no cérebro estarem inacessíveis à maioria dos fármacos e, como consequência, os parasitas conseguem voltar à corrente sanguínea. Dados do nosso laboratório mostraram que o tecido adiposo também poderá ser um importante foco de recidiva e imagens obtidas através de modelos animais revelaram a presença de parasitas no abdómen inferior de ratinhos macho, sugerindo que os tripanosomas poderão persistir nos testículos.

O trato reprodutor masculino é constituído por testículo, epidídimo, vaso deferente, glândulas sexuais acessórias e pénis. Os testículos estão envolvidos pela túnica vaginal numa camada dupla, exceto nas extremidades superior e posterior, onde o cordão espermático e o epidídimo aderem aos testículos; sob a túnica vaginal, há outra camada - a túnica albugínea, que é uma cobertura externa fibrosa e resistente do testículo. O epidídimo é uma estrutura alongada, localizada na borda posterior do testículo. É composto de 3 partes, incluindo a cabeça, corpo e cauda. Devido ao seu comprimento, o ducto epididimal permite espaço para armazenamento e maturação dos espermatozóides. A presença de tripanosomas nos testículos poderá ser uma fonte adicional de recidivas, uma vez que este órgão está equipado com uma barreira hemato-testicular. Esta barreira visa proteger as células germinais de influências prejudiciais e a sua complexidade também impede a entrega de muitos fármacos quimioterápicos para as células testiculares.

O modo clássico de transmissão da tripanossomíase é através da picada de uma mosca, (Tsé-tsé), contrastando com a transmissão vertical através do leite de fêmeas lactantes, observado para *T. evansi*, ou transmissão sexual, em *T. equiperdum*. Uma vez que há

evidências epidemiológicas da transmissão sexual de *T. brucei* em seres humanos, perceber como os tripanosomas estão distribuídos no sistema reprodutor masculino também pode ajudar a identificar os caminhos subjacentes à transmissão sexual da doença.

Neste trabalho caracterizámos a infecção no sistema reprodutor masculino de ratinho utilizando um modelo animal. Para determinar a localização anatómica exata de *T. brucei* nos órgãos reprodutores, utilizámos o modelo de ratinho bem estabelecido C57BL/6J infectados com o clone pleomórfico AnTaT 1.1^{E}. Utilizando rotina histológica e imunohistoquímica, avaliámos a distribuição e a densidade de parasitas em diferentes dias após a infecção. No dia 6 após infecção, observaram-se parasitas no tecido adiposo do epidídimo, de acordo com nosso trabalho anterior. Nesse estadio de infecção também foram detectados parasitas na túnica albugínea e no espaço intersticial/estroma do epidídimo. Contrariamente ao que está descrito na literatura, no nosso modelo não encontrámos parasitas no estroma do testículo. A topografia da distribuição do parasita é mantida ao longo da infecção, sempre com nenhuma ou mínima infiltração do testículo. Para quantificar os parasitas, utilizou-se DNA genómico de *Trypanosoma* (gDNA), que foi quantificado aos 6 e 27 dias após infecção. Tanto no testículo como no epidídimo, a quantidade de parasitas aumentou ao longo da infecção. Quando comparámos esses órgãos em cada dia de infecção, observámos que a quantidade de parasitas no epidídimo é maior do que nos testículos, o que é consistente com as nossas observações de imunohistoquímica. Uma vez que o interstício do epidídimo, a túnica albugínea e o tecido adiposo do epidídimo se encontram fora da barreira hemato-testicular, concluímos que a grande maioria dos tripanosomas que se infiltram no sistema reprodutor masculino não estão protegidos do sistema imunológico do hospedeiro nem de fármacos.

Em seguida avaliámos a extensão e a dinâmica da resposta imunológica nos órgãos reprodutores masculinos usando imunohistoquímica. No dia 6, não foram observadas células inflamatórias. A partir de dia 13, observámos um infiltrado abundante em células mononucleares, juntamente com parasitas. Entre os dias 27 e 41 estavam também presentes granulomas. Para testar o impacto da resposta imune sobre os parasitas, utilizámos microscopia eletrónica de transmissão (TEM) nos dias 6 e 27 após infecção. Mais uma vez, os parasitas nunca foram detectados nos testículos. No dia 6, os tripanossomas no estroma do epidídimo apresentaram uma morfologia normal. Em contraste, no dia 27 após infecção TEM revelou que os tripanosomas presentes no

epidídimo tinham graves alterações morfológicas consistentes com morte celular, incluindo a perda de conteúdo citoplasmático e microtúbulos, núcleos isolados, a acumulação de numerosos detritos celulares e fragmentos de flagelos. Estes resultados sugerem que, nos órgãos reprodutores masculinos, os parasitas estão associados a uma resposta inflamatória que é parcialmente eficaz na eliminação do agente infeccioso e, consequentemente, numa fase tardia da infecção, podem ser encontrados numerosos parasitas mortos, especialmente no estroma do epidídimo.

Os granulomas têm sido descritos como lesões comuns durante a tripanossomíase, em humanos e em numerosas espécies animais. No nosso estudo, foi possível confirmar esse tipo de lesão, ainda que nos estadios finais da infecção. Estes granulomas consistiram em lesões inflamatórias causadas por lesões nas células epiteliais dos túbulos e ductos, com vazamento dos espermatozóides para o espaço intersticial. Como o espermatozóide é antigenicamente estranho, provoca uma inflamação granulomatosa rica em macrófagos e células gigantes multinucleadas. Este dano nos ductos epididimais é supostamente associado à presença de tripanossomas, mas a inflamação granulomatosa que surge como consequência desse dano é dirigida para os espermatozóides, não para os parasitas. Os granulomas de esperma são vistos apenas na fase crónica da doença, mas demonstrámos que nessas fases tardias da doença já existe uma morte parasitária maciça no epidídimo, o que implica a existência de uma resposta imune específica do parasita montada anteriormente. Estas observações sugerem que a resposta inflamatória grave e consequente lesão tecidual e quebra das barreiras teciduais nos órgãos reprodutores podem tanto prejudicar a libertação do fármaco como favorecer a passagem de tripanossomas para os túbulos/ductos do epidídimo, permitindo a transmissão sexual.

Este trabalho abre caminho para pesquisas futuras, com o objetivo de determinar a causa do tropismo parasitário nos órgãos reprodutores masculinos, estudos de difusão de fármacos, persistência de parasitas e transmissão sexual em doenças crónicas e desenvolvimento de fármacos novos e mais eficientes para combater a infecção em todos os compartimentos anatómicos. Além disso, como o controlo do vetor Tsé-tsé atualmente é o foco principal da prevenção da doença, encontrar novas estratégias para diagnosticar e tratar a doença do sono que não requerem o vetor pode ter consequências epidemiológicas globais. Durante a infecção crónica, a carga parasitária é muito baixa e flutua diariamente. É difícil determinar a falha do tratamento uma vez que nem a PCR nem a detecção de anticorpos a partir de amostras de sangue são suficientemente

sensíveis. O uso de imunohisto/citoquímica de biópsias por aspiração com agulha fina poderá ser equacionado como um teste alternativo para o diagnóstico de tripanossomíase.

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Introduction

Human African trypanosomiasis (HAT), also known as sleeping sickness is caused by the protozoan parasite *Trypanosoma brucei gambiense* in West and Central Africa and *T. b. rhodesiense* in East Africa. *T. b. gambiense* is responsible for about 97% of the cases in humans, while *T. b. rhodesiense* is more rare but causes a more severe disease [1]. In endemic countries, 70 million people are at risk of *T. brucei* infection [2]. Sleeping sickness progresses in two stages, namely the early (heamolymphatic) stage and the late (encephalic) stage. In the early stage, parasites are present in the blood, the lymphatic system and interstitial spaces. The late stage is characterized by the presence of parasites in the cerebrospinal fluid, which occurs after trypanosome invasion of the central nervous system [2]. HAT diagnosis is based on clinical symptoms and serological screening using card agglutination test (CATT) followed by detection of parasites in blood, lymph or cerebrospinal fluid using microscopic and molecular methods, primarily PCR of the 18S ribosomal RNA gene [3].

A relapse rate of 5-8% has been commonly described but it can reach up to 30% in some important foci of *T. b. gambiense* sleeping sickness [4]. These relapses have been attributed to the fact that parasites present in the brain are inaccessible to most drugs and, as a consequence at a later time, parasites can return to the bloodstream [5]. It remains unknown if the brain is the exclusive source of relapse. Data from our lab has shown that adipose tissue may also be an important source of relapse [6], and imaging data from mouse models infected with luciferase bioluminescent trypanosomes revealed the presence of parasites in the lower abdomen of male mice, which may suggest that trypanosomes persist in the testis [7,8].

The male reproductive tract consists of paired testes, epididymis, vas deferens, the accessory sex glands (seminal vesicles, prostate, bulbourethral glands, ampullary glands and preputial glands, the later two present in rodents but not in man) and penis [9]. What is often anatomically designated as *testis* and is located within the scrotum, corresponds to the external organs of the male reproductive system and includes the paired testes, covered by the tunica albuginea; the epididymis, which is located at the posterior aspect of each testes and is subdivided in head, body and tail; and the epididymal fat body (**Fig. 1A**). The paired testes are mainly composed of seminiferous tubules, lined by Sertoli cells and germ cells, which produce and release the male gametes, spermatozoa; and these tubules are supported by a scant fibrovascular stroma

with interstitial Leydig cells, which secrete hormones (testosterone). The convoluted seminiferous tubules end in tubuli recti, which lead to the rete testis and then over to a collecting chamber to efferent ducts and the head of the epididymis. The major function of the epididymis is the accumulation, maturation, and storage of mature spermatozoa; and the epididymal ducts are composed of a smooth muscle wall and a columnar to cuboidal epithelium, supported by loose fibrovascular stroma.

Due to DNA exchange during the process of meiosis, spermatids and spermatozoa become antigenically foreign, and protection of sperm antigens from the immune system is warranted by specialized barriers: the blood testes barrier (BTB) and the blood-epididymis barrier (BEB) [10,11,12]. Opposed to the BBB, which consists on tight junctions between endothelial cells, further covered by pericytes and astrocytes [13,14], BTB and BEB are not genuine vascular barriers. The primary physical component of the BTB and BEB are the tight junctions formed between adjacent epithelial cells lining the seminiferous tubules (spermatogonia and Sertoli cells) and the epididymal duct (epididymal epithelial cells) [15], which limit movement of sperm antigens out of tubules and ducts, and the ingress of immune cells into their lumen, thus creating an intraluminal immunologically privileged site (**Fig. 1B-C**). Hence, spermatogonia, Sertoli cells and all interstitial cells, including Leydig cells, fibroblasts and endothelial cells, reside outside of the protective BTB. When there is disruption of the seminiferous or epididymal epithelia, the spermatids and spermatozoa that are normally protected by the BTB and BEB are capable of eliciting an inflammatory response and form sperm granulomas. This consists in a foreign body reaction, a chronic inflammatory lesion macrophage-rich, which in severe/chronic cases can lead to male sterility. Sperm granulomas commonly develop as a sequel to an earlier interstitial inflammatory response, frequently caused by microbial infections [16].

The classical mode of transmission for *T. brucei* is through bite of a biological vector, the tse-tse; which contrasts with the vertical transmission through milk of lactating females, seen for *T. evansi*, or sexual transmission, in *T. equiperdum*. There is however epidemiological evidence in humans, and also data from experimental models, in rodents, for the sexual transmission of *T. brucei* [8,17]. Biologically, for this to be achieved, the parasite would have to be present in the semen; meaning that trypanosomes would have to migrate from the blood to the stromal compartment of the testis and/or epididymis, and from there invade the tubules and/or ducts of the reproductive tract.

Disease pathology often correlates with sites of accumulation of the infectious agent within its host, including the brain, which is associated with characteristic neuropsychiatric symptoms and sleep disorder. Impotence and sterility are other occasional clinical signs of sleeping sickness pathology, but the mechanisms underlying those features are scarcely studied [18,19]. Here we characterize the distribution of *T. brucei* parasites in the male reproductive organs of infected mice. We found an accumulation of both parasite and inflammatory cells in the reproductive organs. Our findings suggest that the inflammation seen in the reproductive organs may compromise proper drug diffusion in the tissue, thus reducing drug treatment efficacy. Furthermore, the high number of parasites seen in the epididymis may allow sexual transmission, and therefore reproductive organs may represent an anatomical reservoir of infection.

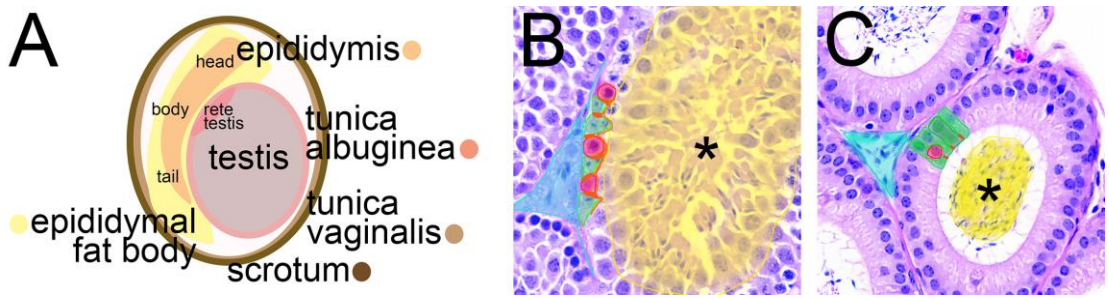

Figure 1. Male reproductive system anatomy, histology and local tissue barriers. **A**. External organs of the male reproductive system: testis, epididymis, epididymal fat body (adipose tissue), fibrous tunicas, albuginea and vaginalis, and scrotum (skin) that encloses all organs. **B**. Histologically the testis are composed by seminiferous tubules, lined by the spermatogonia (red) and Sertoli cells (green), and by the spermatids and spermatozoa (yellow); and these tubules are supported by the stromal compartment (interstitium, blue), rich in connective tissue, vessels and Leydig cells. **C**. The epididymis consist in ducts lined by a monolayer of epithelial cells (green), whose lumen contains numerous spermatozoa (yellow), also supported by the stromal compartment (interstitium, blue); lymphocytes (red) are occasionally seen to infiltrate the basal half of the intercellular space of the epithelia. The immunologically privileged compartments within these organs correspond to the luminal spaces of the tubules and ducts (in yellow), protected by the blood-testis, and blood-epididymis barriers, respectively, which consist in tight-junctions (orange) between Sertoli and spermatogonia, in the testis, and apical cytoplasmic membrane between epithelial duct cells of the epididymis. *Hematoxylin and eosin, original magnification 40x (B, C).*

Methods

Animal Experiments

All animal experiments were performed according to EU regulations and approved by the Animal Ethics Committee of Instituto de Medicina Molecular (IMM), (AEC_2011_006_LF_TBrucei_IMM). The animal facility of IMM complies with the Portuguese law for the use of laboratory animals (Decreto-Lei 113/2013); and follows the European Directive 2010/63/EU and the FELASA (Federation of European Laboratory Animal Science Associations) guidelines and recommendations concerning laboratory animal welfare. All infections were performed in wild-type male C57BL/6J mice, 6–10 weeks old (Charles River, France), by

intraperitoneal injection of 2,000 *T. brucei* AnTat 1.1E 90-13 parasites. For parasite counts, blood samples were taken daily from the tail vein. Organs/tissues of infected mice were collected at days 6, 13 and 27 post-infection. Collected organs were snap frozen in liquid nitrogen or fixed in 10% neutral-buffered formalin. Animals were sacrificed by CO_2 narcosis.

Histology and Electron Microscopy

Mice were killed with CO2 narcosis and the testis and epididymis were colected, formalin-fixed and paraffin-embedded. 4um sections were stained with Hematoxylin and Eosin and were immunostained with a non-purified rabbit serum anti-*T. brucei* VSG13 antigen (crossreactive with many VSGs) and a non-purified rabbit serum anti-T. brucei H2A. Analysis was performed in a Leica DM2500 microscope coupled to a Leica MC170 HD microscope camera

For Transmission Electron Microscopy, samples were fixed with a solution containing 2.5% glutaraldehyde (Electron Microscopy Sciences, EMS) plus 0.1% formaldehyde (Thermo Fisher) in 0.1 M cacodylate buffer (Sigma), pH7.3 for 1 h. After fixation, samples were washed and treated with 0.1% Millipore filtered cacodylate buffered (Sigma), post-fixed with 1% Millipore-filtered osmium tetroxide (EMS) for 30 min, and stained en bloc with 1% Millipore-filtered uranyl acetate (Agar scientifics). Samples were dehydrated in increasing concentrations of ethanol, infiltrated and embedded in EMBed-812 medium (EMS). Polymerization was performed at 60°C for 2 days, and ultrathin sections were cut in a Reichert supernova microtome, stained with uranyl acetate and lead citrate (Sigma) and examined in a H-7650 transmission electron

microscope (Hitachi) at an accelerating voltage of 100 kV. Digital images were obtained using a XR41M mid mount AMT digital camera (Advanced Microscopy Techniques Corp).

Parasite quantification

Collected organs were snap frozen in liquid nitrogen. Genomic DNA (gDNA) was extracted from 25 mg of tissue using NZY tissue gDNA isolation kit (NZYTech, Portugal). A standard curve for quantification of parasite number was obtained by quantifying 18S rRNA gene of *T. brucei* from gDNA extracted from four serially diluted independent cultures. Quantitative PCR (qPCR) was performed on an ABI StepOnePlus real-time PCR machine and data was analyzed with the ABI StepOne software.

Statistical analysis

Statistical analyses were all performed in the free software R: http://www.rproject.org . Statistical analyses were performed by fitting LME models with mice as random effects unless otherwise indicated. At least two independent experiments were considered in each case and statistical significance was set to a = 0.05 level. Data were analyzed after logarithm transformation.

Results

Parasites reside outside the blood-testis and the blood-epididymis barriers

In order to determine the exact anatomic location of *T. brucei* in the reproductive organs and to assess whether it changes during infection we used the well-established mouse model of C57BL/6J mice infected with the pleomorphic clone AnTaT 1.1^{E} [6]. Using histological and immunohistochemistry approaches, we assessed parasite distribution and density at different time post-infection. On day 6 post-infection, parasites were observed in the epididymal fat body, in agreement with our previous work demonstrating that the adipose tissue is a major reservoir for trypanosomes, and that *T. brucei* accumulates in this tissue very early upon infection [6]. At this early time-point parasites were also detected in the tunica albuginea (fibrous capsule that encloses the testis), and the interstitial space/stroma of the epididymis (**Fig. 2**). Contrary to previous descriptions, in our model we did not find parasites in the stroma of the testis, nor in the duct and tubular luminal compartments of epididymis and seminiferous tubules of the testis, respectively. On days 13, 20, 25, 27 and 41 post-infection, the topography of parasite distribution is maintained throughout infection, always with minimal or no infiltration of the testis (tubules and stroma).

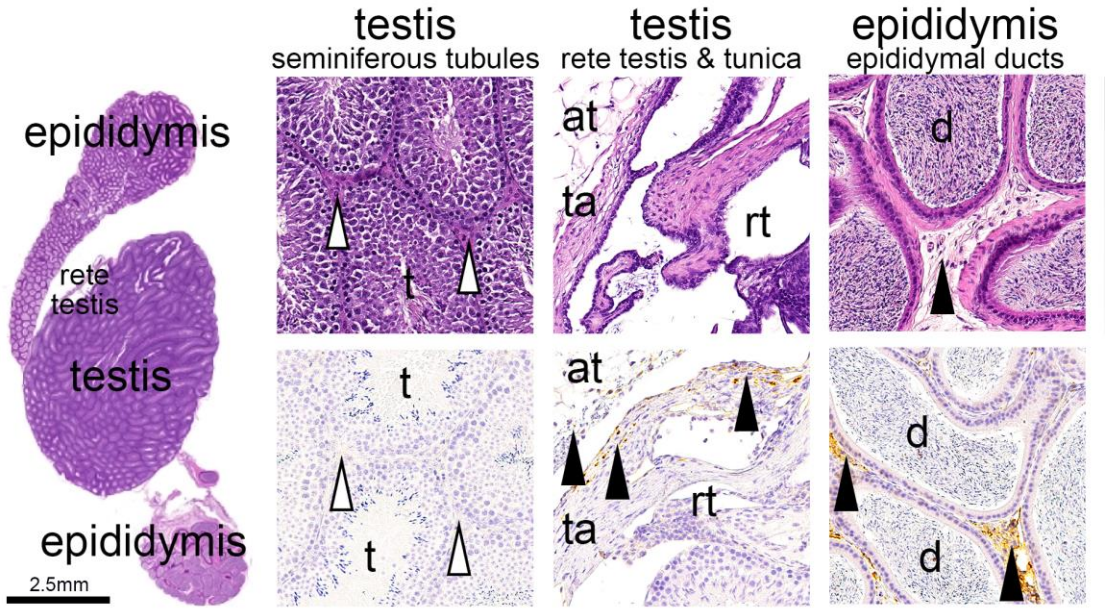

Figure 2. Representative microphotographs of epididymis and testis, at day 6 post-infection with *T.b.brucei*. No parasite infiltration was seen in the testis, inside tubules (**t**) or in the stromal compartment (**white arrowhead**); but numerous parasites (**black arrowhead**) were seen in the adipose tissue (**at**) and tunica albuginea (**ta**), adjacent to the rete testis (**rt**), The interstitial tissue/stroma surrounding the

epididymal ducts (**d**) also shows moderate to marked parasite infiltration (**black arrowhead**). Trypanosomes can be observed in routine histology (upper panel) but are especially noticeable in sections immunostained for VSG (lower panel). *Hematoxylin and eosin (upper panel); anti-VSG, DAB counterstained with Harris hematoxylin (lower panel); original magnification 40x*.

To quantify parasite density, we used as a proxy Trypanosome genomic DNA (gDNA), which was quantified at 6 and 27 days post-infection (**Fig. 3)**. In both testis and epididymis, parasite density increases with time (LME, P<0.0001). When we compare these organs at each day of infection, we observed that parasite density in epididymis is higher than in testis (LME, P<0.0001), which is consistent with our observations of immunochemistry.

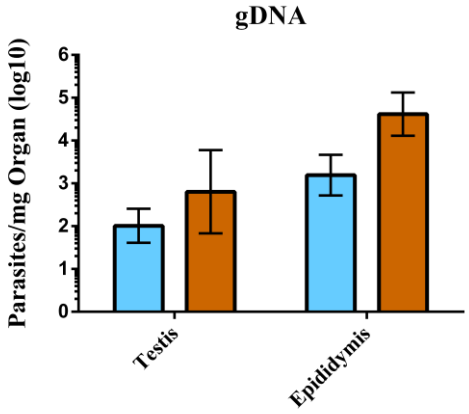

Figure 3. Parasite density in testis and epididymis at days 6 and 27 post-infection. Parasite genomic DNA (gDNA) quantification shows that parasite load increases with time. Represented are the geometric means and the respective standard errors. Blue – day 6; orange - day 27.

Given that the interstitium of the epididymis, the tunica albuginea and the epididymal adipose tissue reside outside the blood-testis and the blood-epididymis barriers (BTB, BEB), we conclude that the great majority of the trypanosomes that infiltrate the male reproductive system are not protected from the immune system nor trypanocidal drugs.

Inflammatory response in male reproductive organs

Using the same histological slides, next we evaluate the extent and dynamics of the immune response in the male reproductive organs. On day 6, inflammatory cell infiltration was not seen. From day 13 onward, we observed a mononuclear-cell rich

infiltrate, interspersed with the parasites; and from days 27 to 41 sperm granulomas (**Fig. 4**) were also present (described in detail below).

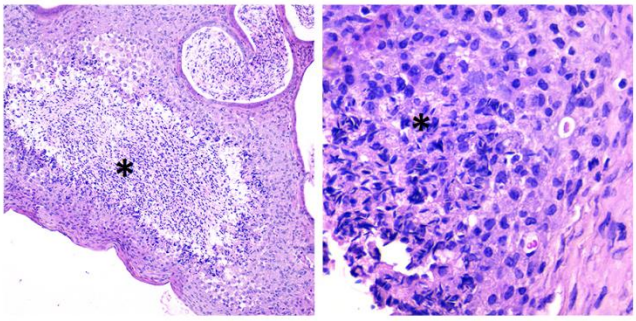

Figure 4. Severe inflammatory cell infiltration seen later in infection. In epididymis, the massive parasite infiltration was associated with inflammatory cell infiltration, mononuclear cell-rich also associated with sperm granulomas. Asterisk, inflammatory cell infiltrates. *Original amplification 10x (A) and 40x (B and C).*

To test the impact of the immune response on the parasite population, we performed transmission electron microscopy (TEM) 6 and 27 days post-infection. Once again, parasites were never detected in testis on any of the time-points. On day 6, trypanosomes in the stroma of the epididymis presented a normal morphology: the microtubule cytoskeleton extends from the basal body to the kinetoplast, we can discern several endosomal compartments and the single Golgi complex are located within the posterior part of the cell between the flagellar pocket and the nucleus. In contrast, on day 27 post-infection TEM revealed that in the epididymis trypanosomes had severe morphological changes consistent with cell death, including loss of cytoplasmic content and microtubules, isolated nuclei, accumulation of numerous cell debris and fragments of flagella (**Fig. 5**).

Together, these results suggest that, in the male reproductive organs, parasites are associated with an inflammatory response that is somewhat effective in eliminating the infectious agent and, as a consequence, later in infection, numerous dead parasites can be found, especially in the epididymis stroma.

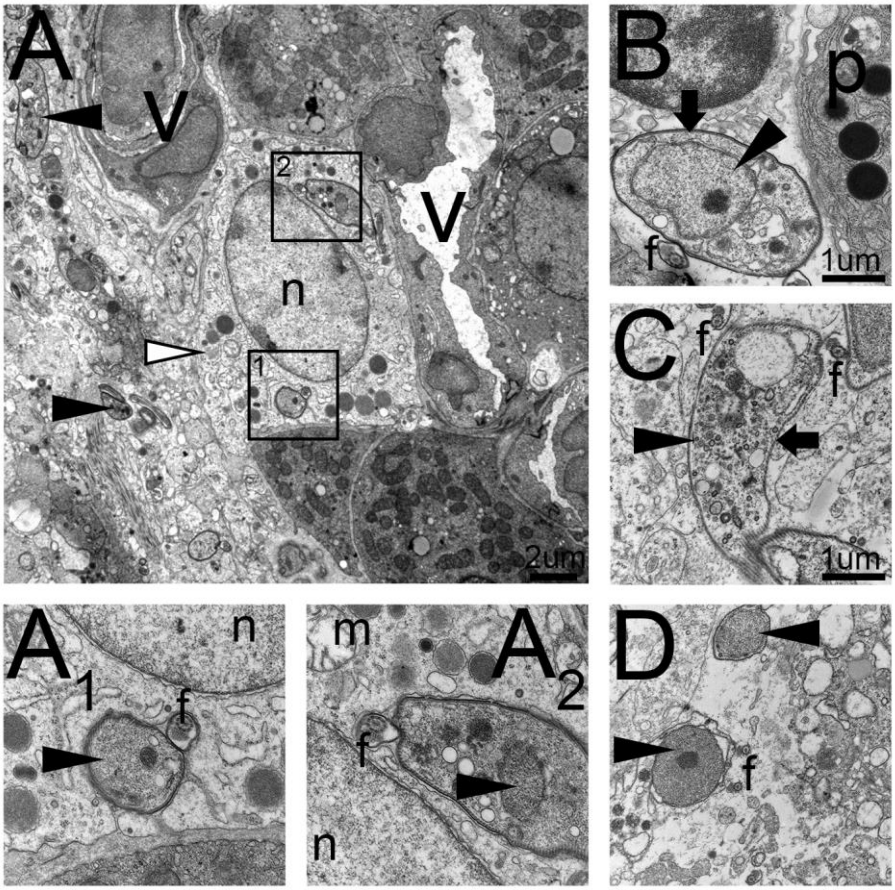

Figure 5. Representative transmission electron micrographs of the morphological changes seen in tissue-resident *T.b.brucei*, at late time-points of the infection (day 27). **A**. Intracellular parasites (**insets 1 and 2**), phagocytized by a macrophage (**white arrowhead; n**, nucleus) with perivascular location (**v**, vessel), where extracellular parasites are also visible (black arrowhead), admixed with cell debris and extracellular matrix proteins. **A1 and A2**. These trypanosomes retain most of their morphological features and the **B**. Trypanosome infiltrating the pancreas, adjacent to a pancreatic acinar cell (**p**), with an intact VSG coat (**block arrow**), nucleus (**black arrowhead**) with a single dark nucleoli, and with a flagellum (**f**). These morphological features contrast with those seen for most of the trypanosomes infiltrating the epididymis at day 27 post-infection, that show severe signs of degeneration (**C, D**); there is loss of the VSG coat (**block arrow**) and disruption of the cell membrane enclosing cell body; and the flagella (**f**) and nuclei, occasionally with clear nucleoli, are often the only organelles visible in the tissue (photo **D, black arrowhead**).

Reproductive pathological consequences of *T. brucei* infection

Granulomas have been described as common lesions during trypanosomiasis, in humans and numerous animal species [20]. In our study, we were able to confirm this type of lesion, albeit at late stages of the infection. From days 27 to 41, sperm granulomas were detectable (**Fig. 4**). These granulomas consisted on an inflammatory lesion caused by damage to the epithelial cells of the tubules and ducts, with leakage of the spermatozoa

to the interstitial space. As sperm is antigenically foreign it will act as foreign-body, leading to a granulomatous inflammation rich in macrophages and multinucleated giant cells. This damage to the epididymal ducts is supposedly associated with the presence of trypanosomes, but the granulomatous inflammation that arises as a consequence of that damage is directed to the spermatozoa, not the parasites. Sperm granulomas are only seen in the chronic phase of the disease, but we have shown that at these late phases of the disease there is already massive parasite death in the epididymis, which entails the existence of a parasite-specific immune response mounted earlier on.

These observations suggest that the severe inflammatory response and consequent tissue damage and breakage of the tissue barriers in the reproductive organs, can both impair the drug delivery and favor the passage of trypanosomes into the tubules/ducts of the epididymis, allowing for sexual transmission.

Discussion

Herein we characterized in detail the presence and distribution of *T. brucei* parasites in the reproductive organs of the male mouse.

No parasites were seen to infiltrate the testis, surrounding the seminiferous tubules or in their lumen, contrarily to what has been suggested [7], On the other hand, we identified numerous parasites in the interstitial space/stroma of the epididymis, with mild infiltration of the tunica albuginea. Concerning the function of this anatomical compartment, the epididymis is known to store spermatozoa for 2–3 months, and during this period the sperm in transit undergoes a maturation process, including gain of mobility, necessary for fertilization of the egg [21]. The severity of epididymal lesions seen upon trypanosoma infection may then reflect the poor quality of semen and high percentage of abnormal spermatozoa present in the ejaculate of trypanosoma-infected bulls [22], goats [23] and sheep [24]. The lesions, resulting from the severe inflammation seen in the epididymis at later stages of the infection, consisted on granulomas, rich in mononuclear cells, which reflects the chronic nature of the infection and the intense antigenic stimulation that exists in epididymal tissue. We can then conclude that trypanosomes infiltrate the male reproductive system and that this may contribute to the impotence and sterility described in sleeping sickness patients [18]. It has also been proposed that the inefficiency of the gonads result from a impairment in the hypothalamic-pituitary-gonadal axis [25], associated with the central nervous system infiltration by parasites, for that more studies are required in order to better understand the pathogenesis of the reproductive disorders in African and Animal trypanosomiasis.

None of the compartments seen to be highly infiltrated by parasites are protected by tissues barriers (BTB or BEB); nevertheless the barriers themselves, especially the BEB, appears to be severely disrupted at late stages of the disease. These observations do not support the hypothesis that parasites are located in immunological privileged sites, and thus protected from drug treatment through this mechanism. Nevertheless, the severe inflammatory response *per se* can not only disrupt the barriers, possibly favoring the passage of trypanosomes into the tubules/ducts, but could also compromise efficient drug diffusion in the tissue and/or protect the parasites admixed in the inflammation, thus reducing drug treatment efficacy.

Another issue also discussed for African trypanosomiasis is the possibility of sexual transmission, which is the primary route of infection for other trypanosoma species, like *T. b. equiperdum* that infects horses. There is however scarce epidemiological evidence in humans for this [17], and few reports validating this hypothesis in animal models [8]. Although it was not possible to analyze the semen of infected mice for parasites, due to experimental constraints, here we observed a large numbers of parasites in the epididymis of all infected mice, which seems to suggest that indeed this distribution pattern may facilitate the passage of these motile parasites into the ducts, favoring sexual transmission.

These studies pave the way for future research, aiming at determining the cause for parasite tropism to these organs, studies of drug diffusion, parasite persistence and sexual transmission in chronic disease and development of new and more efficient drugs to tackle the infection in all anatomical compartments. Furthermore, since control of the tsetse vector is currently the main focus of disease prevention, finding new strategies to diagnose and treat sleeping sickness that do not require the vector, may have global epidemiological consequences. During chronic infection, parasite load is very low and fluctuating daily. Determining treatment failure is difficult since neither PCR nor antibody detection from blood samples is sufficiently sensitive. The use of immunohisto/cytochemistry of subcutis biopsies/fine needle aspiration could be an alternative test for the diagnosis of trypanosomiasis.

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"Só sei que nada sei."

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