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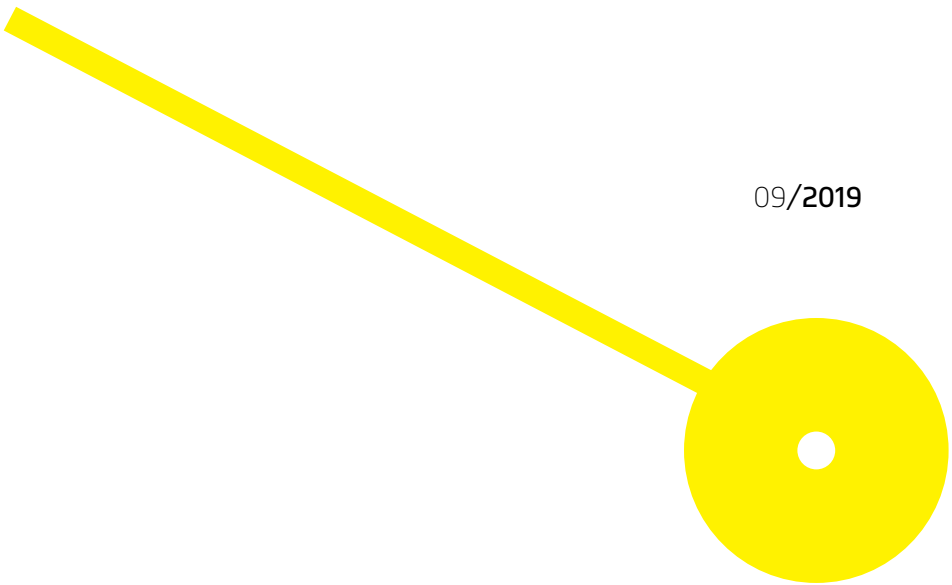
Estrogen Receptor mediated pathway in Schistosomiasis mansoni induced infertility.

Ana Rita Gonçalves Ribeiro



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Estrogen Receptor mediated pathway in schistosomiasis mansoni induced infertility.

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Resumo

Introdução: A schistosomose é considerada a segunda doença parasitária mais devastadora, a seguir a malária, no mundo. Os ovos do schistosoma produzem catecóis-estrógenios. Essas moléculas são metabolizadas em quinonas ativas que causam alterações no DNA. Dado que os estrogénios e os recetores de estrogénios são importantes na reprodução humana, o bloqueio destes recetores por essas quinonas pode afetar a resposta hormonal e, portanto, causar infertilidade. **Objetivos:** Para se compreender melhor esses mecanismos, avaliamos a presença do recetor de estrogénios α (REα) e do recetor 1 de estrogénio acoplado à proteína G (GPER também conhecido como GPR30) nos órgãos de reprodução de um modelo animal infectado com Schistosoma mansoni através de ensaios histoquímicos e imunohistoquímicos. **Métodos:** Na análise histoquímica, foi realizada a coloração com hematoxilina e eosina (H&E). Para a análise imunohistoquímica do REα incubamos secções de tecido com o anticorpo primário monoclonal de coelho anti-REα [SP1], pré-diluído e, para o GPER, incubamos com o anticorpo primário policlonal de coelho anti-GPER. O anticorpo secundário usado tanto para o REα como para o GPER o foi o anticorpo IgG-B biotinilado de cabra anti-coelho. **Resultados:** Existem diferenças histológicas entre os órgãos de ratos infetados e não infetados. A expressão de REα diminui no ovário e aumenta no útero e testículos de ratos infectados com S. mansoni em comparação com os controlos. A expressão de GPER aumenta no ovário e diminui no útero e testículos de ratos infectados com S. mansoni em comparação com os controlos.

Palavras-chave: Schistosomose, infertilidade feminina, infertilidade masculina, estrogénios, recetores de estrogénios, modelos animais

Abstract

Background: Schistosomiasis is considered the second most devastating parasitic disease after malaria in the world. Schistosome eggs produce catechol-estrogens. These molecules are metabolized to active quinones that cause alterations in DNA. Given that estrogens and estrogen receptors are key players in human reproduction, the blockage of Estrogen Receptors by these catechol estrogen quinones might affect the hormonal response and hence cause infertility. **Aims:** To better understand these mechanisms, we evaluated the presence of Estrogen receptorα (ERα) and G protein-coupled estrogen receptor 1 (GPER also known as GPR30) in the reproduction organs of an animal model infected with Schistosoma mansoni through histochemical and immunohistochemical assays. **Methods:** In the histochemical analysis we performed the hematoxylin and eosin (H&E) staining. For the immunohistochemical analysis of GPER we incubated the tissue sections with primary rabbit polyclonal with GPER antibody and for the ERα we incubated with primary rabbit monoclonal [SP1] to ERα, pre-diluted. The secondary antibody was the biotinylated goat anti-rabbit antibody IgG-B and was used for both ERα and GPER. **Results:** There are some histhological differences between infected and non-infected organs from the mice model. ERα expression is decreased in the ovary and increased in the uterus and testes of S. mansoni infected mice compared to controls. GPER expression is increased in the ovary and decreased in the uterus and testes of S. mansoni infected mice compared to controls.

Keywords: Schistosomiasis, female infertility, male infertility, estrogens, estrogen receptors, animal models

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Glossary

Angiogenesis: The formation and development of new blood vessels.

Catechol-estrogen: a steroidal estrogen that contains catechol (1,2-dihydroxybenzene) within its structure. The catechol estrogens are endogenous metabolites of estradiol.

Disability burden: a concept to describe the impact of a condition on the affected population and comprised of a disability (YLD) and a mortality (YLL) component. Estimates of disability burden are used to compare and rank the impact of different conditions.

Dyspareunia: Painful or difficult sexual intercourse, especially in women.

Dysuria: The experience or condition of experiencing pain while discharging urine.

Fistula: An abnormal connection or passageway between organs or vessels that normally do not connect.

Funiculitis: inflammation of a funiculus, especially of the spermatic cord.

Hematospermia: The presence of blood in the semen.

Hematuria: The presence of blood in the urine.

Mass spectrometry: an analytic technique by which chemical substances are identified by the sorting of ions in electric and magnetic fields according to their mass-to-charge ratios.

Mesenterium: a contiguous set of tissues that attaches the intestines to the posterior abdominal wall.

Metabolite: a chemical substance that is a product of metabolic action or that is involved in a metabolic process.

Neglected tropical diseases (NTDs): a diverse group of 20 communicable diseases recognized by WHO as being underfunded relative to other conditions of equal prevalence; NTDs affect predominantly the world's poorest populations, and many have a chronic course, are physically disabling, and/or are associated with stigmatization.

Pampiniform plexus: a network of many small veins found in the human male spermatic cord.

Plexus: a branching network of the vessels or nerves.

Quinones: oxidized derivatives of reactive aromatic compounds with electron-donating substituents such as catechols.

Seminal vesiculitis: an uncommon entity characterized by inflammation of the seminal vesicles. It is most commonly infective in etiology and often associated with concurrent infection elsewhere in the male genital tract.

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1. Introduction

1.1 Global schistosomiasis status.

The neglected tropical diseases (NTDs) are a group of chronic, disabling, and disfiguring conditions that occur most commonly in the setting of extreme poverty, especially among the rural poor and some disadvantaged urban populations [1]. One of these NTDs, schistosomiasis, is sometimes referred to as bilharzias, bilharziasis, or snail fever. Schistosomiasis was discovered by Theodore Bilharz, a German surgeon and is considered a parasitic helminthic NTD. Like many tropical diseases, is endemic in areas where poor living conditions and poverty are prevalent. Because these are water-borne species, populations that primarily rely on agriculture and fishing for their livelihood are at the highest risk of contamination [2–4]. Schistosomiasis remains a major public health problem. It is endemic in 75 countries, and most cases of schistosomiasis occur in Africa, making this condition one of the most common NTD in the region [5–7].

The World Health Organization estimates that schistosomiasis affect at least 220,8 million people worldwide. This disease can cause chronic anemia and inflammation associated with severe disability burden among children, adolescents, and young adults and has an incidence of 200,000 deaths yearly [8,9]. Trapped ova induce a chronic granulomatous response that causes local and systemic pathological effects ranging from anemia, growth stunting, impaired cognition and decreased physical fitness, with largely unknown effects on endocrinologic hormones and their receptors to organ-specific effects such as severe hepatosplenism, portal fibrosis with portal hypertension [3,9,10].

1.2 Schistosomiasis and Infertility

Infertility is defined as the incapacity to become pregnant after 12 months of regular and unprotected intercourse. This is a major public health problem that affects about one in six couples (15-20%) worldwide and 12–30% of couples in sub-Saharan Africa[11–13]. It is well recognized as a major sequela of genital tract infection, many women in Africa with this ailment (85% to 90%) suffer from subsequent infertility, and the cause can be attributed to pelvic infections[14]. Numerous diseases including febrile diseases, sexually transmitted infections (STDs) and various parasitic infections, such as microfilaria and schistosomiasis, can cause infertility[15].

When considering the diagnosis of infertility there are two main features of this disease: primary infertility considered when the woman never conceived, and secondary infertility when she

had had a previous labour [15]. While most papers addressing infertility associated-schistosomiasis only describe primary infertility we found only two reports in literature describing secondary infertility: one from our group [15] and another one [16] both reporting a lack of association between secondary infertility and schistosomiasis.

1.3 Epidemiology and pathogenesis of genital manifestations of schistosomiasis

As stated, schistosomiasis affects millions of people worldwide. About 20 million have severe disease and the risk of infection affects 600 million others, including travelers from developed countries [17–19]. Both sexes can develop genital tract pathologies, but the prevalence is significantly higher in women. [20,21]. The pathogenesis of this disease in the genital tract is thought to be caused by ova deposition and ensuing inflammation causing mechanical blockage, scar tissue and destruction of anatomical structures [22,23]. Infection of the genital tract is most commonly caused by the S. haematobium species. The eggs are laid by adult worms residing in vasculature and they migrate to the bladder, and it is in this organ that the main pathology occurs. However, S. haematobium eggs can be deposited along the genitourinary tract such as the cervix and vagina in women, and testes in men, due to the proximity of genital venous plexus (see Glossary), which allows easy parasitic migration. [5,6,10,24,25].

Schistosomiasis is not essentially a thromboembolic disease (i.e., defined as embolic events within the vasculature, but its eggs may lead to occlusion of the terminal veins and impairment of the blood supply [26]. It is widely described as a granulomatous disease with eggs becoming lodged in tissue and then leading to inflammatory reactions and fibrosis. It is believed that infertility could be related to this tissue scarring, ensuing inflammation and granulomatous reactions, due to the release of proteolytic enzymes by the ova, causing mechanical blockage, scar tissue and destruction of anatomical structures [22,27,28]. As mentioned, women's infected ovaries can become scarred and fibrous, and Fallopian tubes may show nodular lesions. Therefore, this fibrosis of the ovaries and/or tubal occlusion caused by granulomas, is thought to lead to infertility [16,29,30] . This could be explained by the egg granuloma composition of eosinophils, mononuclear phagocytes, fibroblasts, lymphocytes, neutrophils, plasma cells and mast cells. The systemic immune response is triggered by cercariae, adult worms and eggs and their antigens are transported to lymphoid organs, where they are captured, processed and presented to antigen specific T-cells which leads to T-cell activation and lymphokine secretion. Thus, systemic activation of immune responses ensues, triggering this way the chronic fibrosis, tubal adhesions and possibly infertility. Anything, which leads to an inflammatory response such as an infection in the pelvis, pelvic inflammatory disease, or even a ruptured appendix, can trigger adhesion formation. Adhesions, also called scar tissue, can

block or distort the fallopian tubes [16,29,31]. This could also explain infertility associated to S. mansoni, characteristically laying its eggs on the mesenterium plexus, and therefore not be able to cause infertility by direct or mechanical cause. In fact, our group has demonstrated that mice infected with S. mansoni have impaired reproduction by diminishing gestational length, number of pups and histopathological alterations in reproductive organs [32].

In men, the symptoms include epididymitis (an inflammation of the epididymis at the back of the testicle) which can simulate tuberculosis and associated funiculitis, indolence and possible formation of a fistula, hematospermia, pain during urination, prostatitis and others. In men, reproduction is affected by schistosomiasis by predisposition to secondary infection of the prostate and seminal vesicles. Sometimes it directly affects the testicles. Being this a solid organ, the trapped eggs induce a granulomatous reaction, which leads to fibrosis in the case of S. haematobium infection [20,21] and metaplasia and necrosis in the case of S. mansoni infection [32].

Genital schistosomiasis is also linked to horizontal transmission of human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS). The presence of eggs in the Lower Genital Tract, that includes the cervix, vulva, and vagina, give rise to a wide array of symptoms in the affected women, and this lesions, if located in the vagina or vulva, might lead to hypertrophy, and can grow for months or years. Consequently, the capacity of the epithelium to act as a physical barrier to viral transmission is probably reduced leading to the possibility of transmission and propagation of HIV. This happens because vaginal and cervical lesions tend to bleed easily, therefore the HIV present in semen would have direct access to the blood circulation and, through via ulcerative lesions, to the regional lymph nodes [33,34].

Hybrid schistosomes are particularly worrying. Experimental studies have revealed that interspecific hybridization might enhance infectivity and virulence. Also, hybrids can have wider host spectrums, potentially expanding their epidemiologic consequences [35]. At present, there isn´t any report addressing hybrids associated to infertility but given the fact that these hybrids give rise to characteristically ectopic egg locations it might increase association of S. mansoni infertility both in females and males.

1.4 Mechanisms underlying female and male infertility-associated schistosomiasis

Female

Schistosomiasis haematobia remains a significant risk factor because several cases of infertility in schistosomiasis endemic areas have been attributed to female genital schistosomiasis (FGS) [13,36]. FGS is defined as the existence of schistosome eggs in the female reproductive system. The eggs cause chronic inflammation in the surrounding genital tissue, comprised of granulomas,

fibrosis, and angiogenesis. Together with this, girls and women can experience hematuria, dysuria, urinary frequency, and an increased risk of bladder cancer [22]. "Classical" lesions of female genital schistosomiasis, which consist of eggs and granulomas associated with increased angiogenesis and inflammation, are described as "sandy patches". Sandy patches usually bleed on contact and can cause genital itching and pain, stress incontinence, dyspareunia and possibly infertility [5].

FGS can be associated with endometritis and is likely to cause female infertility. Urogenital schistosomiasis, in individuals of reproductive age, remains highly prevalent and underdiagnosed [3,6]. Lesions in the upper genital tract, which includes the uterus, fallopian tubes and ovaries, are less discernible by routine clinical examination [33]. Fibrosis of the ovaries or tubal occlusion caused by granulomas, due to ovarian, tubal and uterine schistosomiasis, are thought to lead to infertility (Table 1)[33,34]. Schistosomiasis mansoni is also likely to cause infertility in women although there are very few reports in the literature. In fact there are only two reports describing ectopic location of S. mansoni eggs in endocervix [3] and in endometrium and ovary [37] and another two reports describing hormonal imbalance in female mice infected with S. mansoni [38,39] . Hormonal disturbances may also explain female infertility and subfecundity associated to S. mansoni infection (Table 1) [12,15,32].

Male

Male infertility due to schistosomiasis haematobia, unlike female infertility, is poorly reported and can develop through different mechanisms: 1) Testicular, in which there is a direct damage of testicular tissue by bilharzial inflammation and granuloma formation; 2) Post-testicular, due to obstruction of excurrent genital ductal system at any region between the rete testis and ejaculatory ducts, and / or affection of the accessory sex organs (prostate, seminal vesicles) by bilharzial inflammation and granuloma formation, resulting in severe oligozoospermia (partial obstruction), azoospermia (complete obstruction) or subfertile semen parameters [40–43].

Worms can reach the veins of the spermatic cord, epididymis, and testis through several routes: a) connection between the superior mesenteric and right spermatic veins, or along those between the inferior mesenteric and left spermatic veins; b) connection between the deferential vein and pelvic veins; c) connection between pampiniform plexus in the scrotum and pelvic veins that cross near the external inguinal ring; d) direct spread from the epididymis to the testis (possible but remote); e) systemic arterial spread, as observed in cases of ectopic bilharzial lesions (possible but remote) [18].

The trapped Schistosoma eggs, in men, leads to occlusion of the spermatic venous plexus and subsequent granuloma formation may result in testicular infarction and, these trapped eggs, may also lead to intense granulomatous epididymitis and inhibition of spermatogenesis causing male infertility [4,42]. Seminal vesiculitis can cause haematospermia, painful ejaculation, burning

micturition and low backache (Table 1) [4,42,43]. Once the disease has progressed to the granuloma stage, the damage is irreversible [42]. Comparable to female S. mansoni infection, there are very few cases of S. mansoni infection causing infertility in men. We found two reports describing ectopic testicular S. mansoni eggs [4,43] and two reports describing male sex hormone imbalance [44,45]. In male animals infected with S. mansoni, again, only two reports describe hormonal imbalance: the work of Lansoud-Soukate et al [46] corroborated recently by our own work [32,39] (Table 1).

1.5 ERs and estrogen metabolism in schistosomiasis

Estrogens exert several biological effects in humans, such as in the cardiovascular, musculoskeletal, immune, central nervous systems and plays an important role in the development and maintenance of normal sexual and reproductive function [47]. Estrogen signaling is primarily mediated through ERs, that incorporate the classic receptors ERα and ERβ and the newly found membrane receptor GPER [48]. ERα is mainly expressed in the gonadal organs (uterus, ovary, prostate, testes, and breast), liver and adipose tissue. ERβ is found mostly in the prostate, bladder, ovary, colon, adipose tissue, and immune system. These classic receptors have common physiological roles such as in the development and function of the ovaries, and estrogen-mediated cardioprotective actions [48,49].

GPER, a member of the G protein-coupled receptor superfamily, has been recognized as a membrane estrogen receptor, which can trigger rapid estrogen non-genomic signaling independent of ERα and ERβ. GPER is expressed in many brain regions, including the hippocampus, cortex, hypothalamus, specific nuclei within the midbrain, and the Purkinje layer of the cerebellum, as well as the adrenal medulla, renal pelvis, and ovaries [48,50]. Many studies suggest that are a number of pathways by which estrogens and their receptors regulate the biological processes.

- **Pathways by which estrogens and their receptors regulate the biological processes.**

The classical pathway includes ligand activation and a directly bind to EREs in target gene promoters which leads to the transcriptional changes in estrogen-responsive genes. Due to the binding of the ligand to the receptor can trigger a recruitment of a variety of co-regulators, which can alter the chromatin structure and facilitated the recruitment of the RNA polymerase II transcriptional machinery [47,48,51,52].

However, ERs can also regulate gene expression without directly binding to DNA. This occurs through protein-protein interactions with other DNA-binding transcription factors in the nucleus. In addition, membrane-associated ERs mediate nongenomic actions of estrogens, which can lead both to altered functions of proteins in the cytoplasm and to regulation of gene expression [47,48,51,52]. The ligand-independent pathway includes activation through other signaling pathways, like growth

factor signaling. In this case, growth factors activate protein-kinase cascades and this activated kinase leads to phosphorylation and activation of nuclear ERs and in that way activate them to dimerize, bind DNA, and regulate genes [47,48,51].

A non-genomic mechanism that have rapid effects, where a receptor is activated by a ligand, possibly associated with the membrane, and the complex formed is associated with the activation of various protein-kinase cascades, leading to indirect changes in gene expression which can alter functions of proteins in the cytoplasm, due to phosphorylation of transcription factors. This rapid effect can cause mobilization of intracellular calcium, generation of cAMP, production of nitric oxide, production, activation of the mitogen-activated protein kinase (MAPK) signaling pathway, activation of the phosphoinositol 3-kinase signaling pathway and activation of membrane tyrosine kinase receptors [47,48,51–53].

Estrogen interacts also with GPER, a third ER discovered more recently, and the stimulation of GPER activates heterotrimeric G proteins, which in turn can activate multiple effectors, resulting in production of cAMP and the activation of Src, and sphingosine kinase. Through Src there are a stimulation of the matrix metalloproteinase (MMP) which leads to a release of HB-EGF that can transactivate epidermal growth factor receptors (EGFRs). The activation of MAP kinases and PI3 kinases, resulting of the activation of EGFR, leads to the expression of transcription factors [50,54]. When classic ERs are absent or blocked the effects mediated by GPERare maintained [12,55].

While addressing schistosomiasis-induced hypogonadism in male patients infected with S. haematobium and S. mansoni, Botelho et al. [45] observed a noteworthy elevation in serum levels of estradiol, whereas those of LH and FSH remained normal, and hypothesized that the excess estradiol could be external to the host. In fact, we found by mass spectrometry that the molecule responsible for the effect was an S. haematobium-derived estradiol-like molecule that is an antagonist of estradiol and thus repressed the transcriptional activity of the ER. Moreover, new estrogenic molecules were identified in S. haematobium total antigen as well as in the serum of infected individuals with this parasitic disease that seem to be produced by this parasite [56]. ER transcriptional activity was suppressed in urothelial cells and ER expression was also suppressed in the bladders of mice in response to S. haematobium [57] .

Schistosome eggs produce a metabolite derived from estrogen called catechol-estrogen. Cytochrome P450 oxygenases metabolize these estrogenic molecules to active quinones, that include the formation of the catechol estrogen-3,4-quinone, the major mutagenic derivative of estrogen, that cause alterations in DNA. [15,58,59]. This ability to cause alterations in DNA is highly inducible of carcinogenesis, in addition we [1] and others [2] demonstrated that these molecules are associated with schistosomiasis- associated bladder cancer as well as thyroid and breast cancer.

In further recent studies, the presence of estrogen-like metabolites during FGS was also associated with self-reported infertility [15]. These metabolites down-regulate ERα and ERβ in estrogen responsive cells in vitro because they can react with DNA to form depurinating adducts, which may generate DNA apurinic sites that, in turn, initiate mutations [57]. Our group demonstrated that the eggs of S. haematobium secrete these novel catechol estrogens, and that estrogens are metabolized to active quinones that modify DNA. Therefore, it is believed that the presence of parasite-derived catechol-estrogens may influence infertility during infection with Schistosoma through the blockage of estrogen receptors acting as endocrine disruptors [15,58,59]. More recently, in order to better understand this hormonal imbalance, our group investigated a serum panel of steroid hormones in Schistosoma mansoni infected female and male hamsters [39].

	Organs affected	Effects	Study model	
			S. haematobium	S. mansoni
Female	Ovaries	Hypogonadism, retarded puberty Infertility (primary/secondary)	Human [15,21–23,26,33,34,45]	Mouse [32,39] Human [37]
	Tubes	Ectopic/tubal pregnancy Tubal abortion Hemoperitoneum	Human [7,13,21,24,26,29,30,33,34,36]	Mouse [32]
	Uterus	Anemia due to chronic blood loss Metaplasia Miscarriage, preterm delivery	Human [16,33,34]	Human [37] Mouse [38]
	Cervix	Anemia due to chronic blood loss Carcinoma Risk for sexually transmitted disease	Human [17,21,25,29,30,33,34,41]	Human [3,34]
	Vagina/Vulva	Destruction of hymen and/or clitoris Vesico-vaginal fistula Risk for sexually transmitted disease	Mouse [10] Human [25,29,30,33,34,41]	
Male	Testicles	Hypogonadism, retarded puberty Infertility (primary/secondary)	Human [19,45]	Human [4,27,43–45] Mouse [32,39,46]
	Epididymis	Pain, funiculitis, fistulization, hemospermia, azoospermia	Human [19,42]	Human [43]
	Prostate	Pain during urination	Human [20]	

Table 1. Effects of schistosomiasis in female and male reproductive organs.

2. Aims and goals

In a previous study that involved residents of rural Angola, a region endemic for schistosomiasis haematobia, authors detected a significant association between the presence of catechol-estrogens/ DNA adducts in the urine of females who were urine egg-positive for S. haematobium infection and self-reported infertility [15].

We now hypothesize the induction of infertility in individuals infected with S. mansoni also through an Estrogen-DNA adduct-mediated pathway. We hypothesize further that the genotoxic effects of estrogen metabolites lead to the formation of estrogen-DNA adducts and hence may be a cause for infertility. The blockage of Estrogen Receptors by these catechol estrogen quinones might also affect the hormonal response and hence cause infertility.

We propose to address these hypotheses through the following specific aim:

Aim: To study Estrogen Receptor α and GPER in the reproductive organs of the animals infected with S. mansoni.

3. Methods

Animal experiments

This experiment followed the guidelines and recommendations of FELASA (Federation of European Laboratory Animal Science Associations) and the European Directive 2010/63/EU related to animal protection in scientific studies, especially the reduction principle of the 3Rs for animal experiments.

Six-week-old CD-1 mice were provided by IBMC (Porto, Portugal).

Animals spent 1 week being acclimated under routine laboratory conditions before starting the experiments. They did not receive any treatment prior the study.

They were fed standard balanced food and water ad libitum. All the animals were maintained at the National Institute of Health (Porto, Portugal) in rooms with controlled temperature (22% 2°C) and humidity (55% 10%) and continuous air renovation. Animals were housed in a 12 h light/12 h dark cycle (8 am–8 pm). All animal experiments were performed in accordance with the National (DL 129/92; DL 197/96; P 1131/97) and European Convention for the Protection of Animals used for Experimental and Other Scientific Purposes and related European Legislation (OJ L 222, 24.8.1999).

Experimental infections

Twelve CD-1 mice from which 8 were experimentally infected with 50 cercariae S. mansoni. These mice were infected by member's extremities and tail immersion, respectively. The control animals consist of 10 littermates. The cercariae were obtained by shedding of snails infected with miracidia.

Necropsy and pathology

Animals were sacrified with the humaine endpoints recommendations by the law 2010/63EU with the respective amandements for the Portuguese law 113/2013. Then, organs were collected, PBS-formalin fixed and stand at least 24hours in this preservative. The organs collected in the necropsy procedures were the gonads of both genders. Female reproductive organs collected include ovaries, oviducts and uterus. Testes were the only reproductive male organ collected.

Then tissues were processed using the standard procedures of dehydratation, clearing (xylene) and wax infiltration with liquid paraffin. After tissue processing blocks were prepared by embedding tissues into a mould with paraffin. Thin sections of 3 μm resulting from microtomey were attached to adhesive prepared slides.

Histochemistry

- **Hematoxylin and Eosin Stain (H&E)**

Blades with tissues sections were deparaffinized in section of xylene 10 minutes, then hydrated in absolute alcohol, 90% and 70% for 5 minutes each. After hydration they were stained in hematoxylin solution for 2 minutes, washed briefly in running water and then washed 5 minutes with 90% alcohol. After these steps they were stained in eosin solution for 1 minute and then were dehydrated through 70% alcohol, 90% and 100%. Lastly were cleaned in xylol for 10 minutes and after this time the blades were mounted with entellan.

Immunohistochemistry

- **Estrogen Receptor α**

Sections of ovaries, uterus and testes were stained for the ERα. Tissue sections were incubated overnight, at 4ºC in a humidified chamber, with a primary rabbit monoclonal [SP1] (ab27595, Abcam plc, UK) to estrogen receptor alpha, prediluted followed by the biotinylated goat anti-rabbit antibody IgG-B (sc-2040, Santa Cruz Biotechnology, Inc., USA; 1:200 dilution).

- **GPER**

Sections of ovaries, uterus and testes were stained for the GPER. Tissue sections were incubated overnight, at 4ºC in a humidified chamber, with a primary rabbit polyclonal to G-protein coupled receptor 30 antibody (ab39742, Abcam plc, UK; 1:200 dilution) followed by the biotinylated goat anti-rabbit antibody IgG-B (sc-2040, Santa Cruz Biotechnology, Inc., USA; 1:200 dilution).

Image Analysis

Histological sections of 4 ovary and uterus tissues and 4 testes tissue and were analyzed with optical microscope Nikon Eclipse 50i (Nikon Instruments Inc.) and images captured with attached Nikon DS-Fi1 digital microscope camera (Nikon Instruments, Inc.), being this camera connected to a computer. Microphotographs of the ovaries, uterus and the testes for each IHC and

HC technique from the mice studied were taken, at 400X optical amplification in Nikon Nis Elements Viewer 3.22.15 (Nikon Instruments Europe B.V.) software. For further analysis, five pictures were obtained for each tissue of most stained areas.

Statistical Analysis

For statistical analysis, with all the collected data, we calculated the mean and the standard deviation (SD). For the hypothesis testing, we first went to determine if we would use a parametric or non-parametric testes, for that we applied the Kolmogorov-Smirnov test to determine if the sample have a normal distribution and Levene's test to check whether two samples have equal variances (homogeneity of variance). Based on that, after this analysis, as our data have a parametric distribution thus it was applied the unpaired t-student test.

The statistical analysis was performed with GraphPad Prism 8, and results were considered statistically significant when p values were < 0.05.

4. Results

In the following pages it will be described the main findings on reproductive organs of CD-1 mice males and females infected with S. mansoni in comparison with non-infected animals. Also, a description on estrogen receptors alpha (ERα) and G-protein coupled ER (GPER) expression by means immunohistochemistry will also be included.

Ovaries

On macroscopic observation, ovaries from infected female appear paler and with more fibrotic aspects on its medullary region.

Regarding microscopic observation in thin-section stained with hematoxylin-eosin (H&E) ovaries of infected females show fibrotic tissue, less vascularization richer in primordial and secondary follicles and showing less mature follicles (tertiary and Graafian follicles) when compared with ovaries from control females.

Immunohistochemistry (IHC) revealed that ERα expression was discrete however control females expressed this receptor on stromal cell from medullar region while infected females' ovaries reveal no expression whatsoever (Figures 1 and 4). On the other hand, GPER stained markedly on infected females' ovaries. The expression was much more intense in both stromal and follicular cells, in particular from the granulosa layer but in lesser extent on the theca cells. Regarding non-infected animals, GPER revealed to be expressed more discretely on all the ovarian tissues (Figure 5), however IHC expression revealed that theca cells around follicles presented quite a GPER expression (Figure 1).

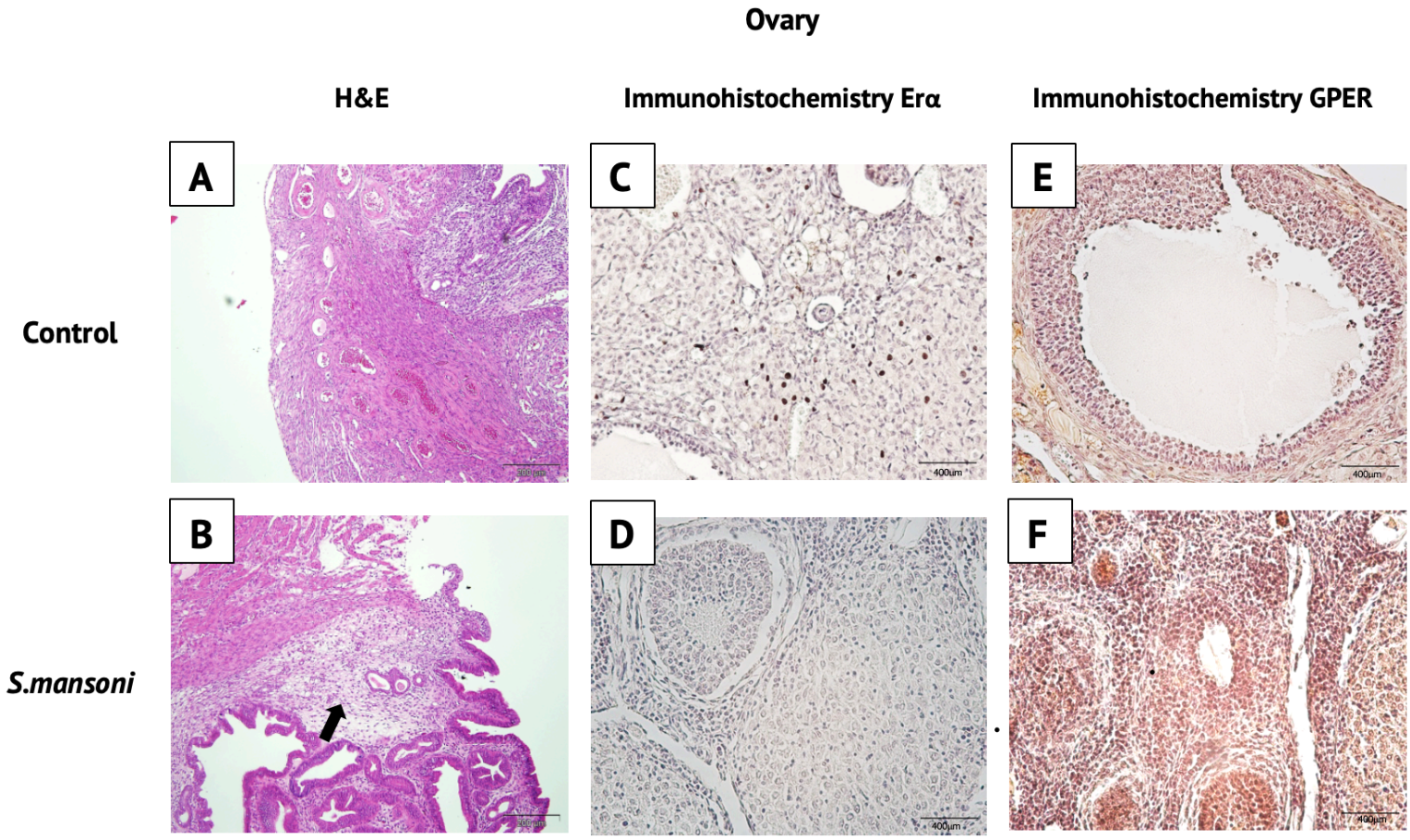

Figure 1.H&E staining and immunohistochemistry (ERα and GPER) of ovary in control vs. infected with S. mansoni mice. H&E of (A) Control and (B) infected ovaries . (B) show fibrotic tissue (arrow). IHC of ERα in (C) Control and (D) infected ovary, the infected ovary shows less or none expression of ERα .IHC of GPER in (E) Control and (F) Infected ovary, (F) shows more expression of GPER, that was especially intense in both stromal and follicular cells.

Uterus

Macroscopically observation revealed the infected animals' uterus were much more fleshy and harder on touch.

When compared to non-infected animals, it was remarkable the hyperplasia presented on epithelial tissue from luminal tubes of the infected females. Besides of significant hyperplasia of lumen epithelial cells, tubes from infected animals presented also an extensive fibrotic tissue in stromal surrounding tubes. Both condition restrained lumen that appears constricted on the organs from the infected females when compared with the same tissues from control females (Figure 2).

Estrogen receptors expression revealed that IHC staining of ERα was modest. Nonetheless, infected mice uterus expressed this receptor on stromal cells while non-infected females' uterus disclosed no expression whatsoever (Figures 2 and 4). Conversely IHC GPER expression was quite opposite. On one hand GPER was expressed quite abundantly in organs from both infected and non-infected females, with a significantly higher expression on infected animals' uterus. On the other hand, expression pattern was also different among infected and non-infected females. The expression on non-infected females was remarkably intense on ciliated column epithelial cells from the luminal portion of the uterus and in lesser extension on the stromal region of the same animals (figure 2).

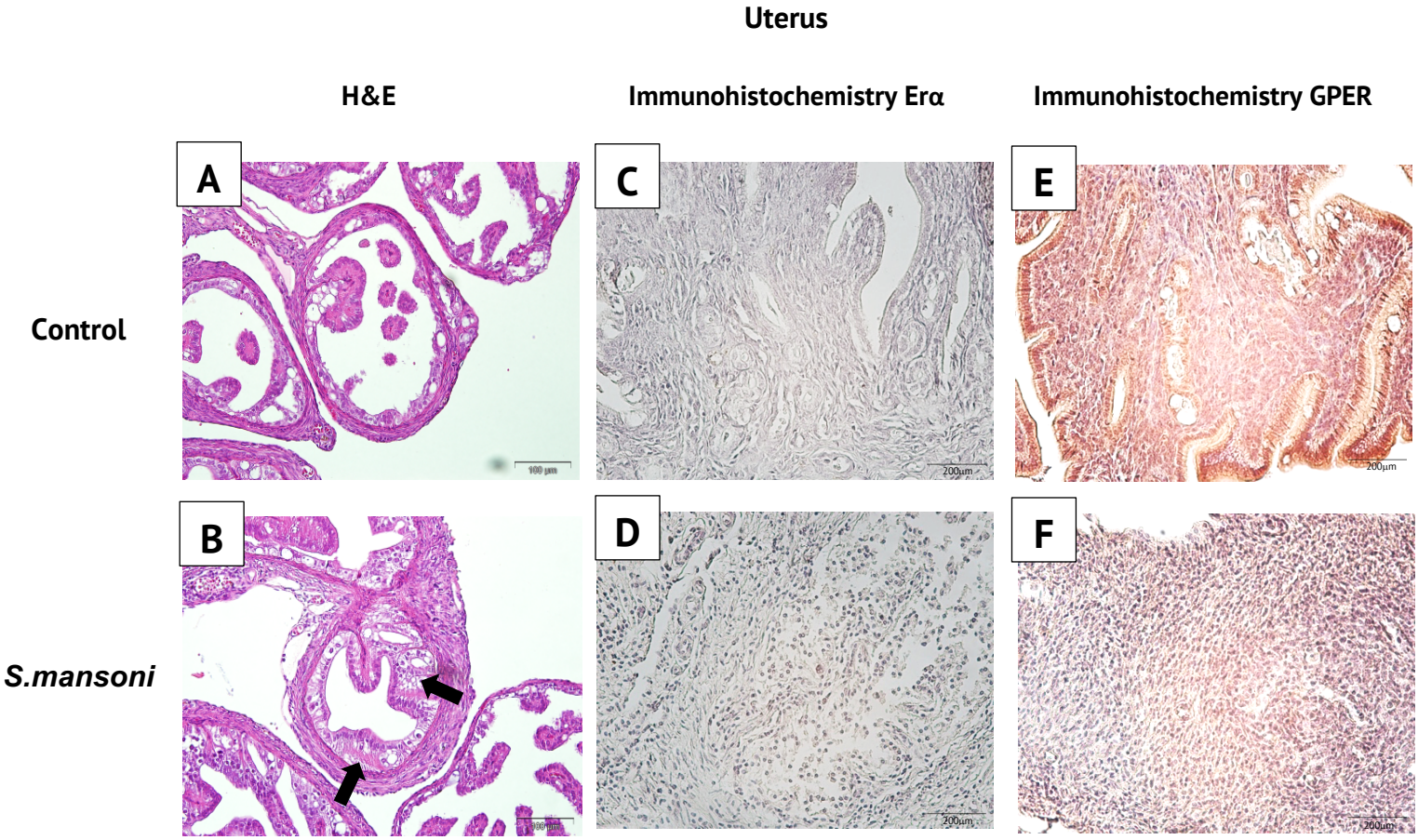

Figure 2. H&E staining and immunohistochemistry (ERα and GPER) of uterus in control vs. infected with S. mansoni mice. H&E of (A) Control and (B) infected uterus. (B) shows significant hyperplasia of lumen epithelial cells (arrows). IHC of ERα in (C) Control and (D) infected uterus. (D) expressed this receptor on stromal cells while (C) showed none or less expression. IHC of GPER in (E) Control and (F) Infected uterus, (E) shows more expression of GPER, expression on non-infected females was more intense on ciliated column epithelial cells from the luminal portion of the uterus.

Testes

On macroscopic observation, each testis from infected male appear smaller and more colored then testes from non-infected males.

Microscopically, H&E indicate that testes from infected mice revealed seminiferous epithelium necrosis with less spermatic cells and remarkable destruction of Sertoli cells when comparing with non-infected animals' testes. Metaplasia of interstitial cells around seminiferous tubes is also observed in comparison with testes from non-infected animals. Hyperplasic and hyperchromatic Leydig cells were also found in testes from infected animals.

When analyzing ERs expression on male gonads, in particular ERα and GPER, Immunohistochemistry revealed, in accordance to what was found on female organs, different expression patterns. First, ERα expression was much more modest than GPER in both infected and non-infected mice (Figure 3). Then, ERα IHC staining was almost absent from non-infected mice but some spermatogonia nuclei expressed such receptor on infected animals. ERα expression is virtually absent in these animals (Figure 4). Regarding GPER expression on non-infected animals' testes was significantly higher when compared with infected animals (Figure 3 and 5). The IHC GPER stain is noteworthy in seminiferous tube's cells, in particular on spermatogonia and Sertoli cells' nuclei however in spermatocytes the localization of the GPER receptors are mainly cytoplasmatic on the non-infected animals. On infected animals, seminiferous tube's cells express significantly less GPER, and it is apparently restricted to spermatogonia. Interestedly Leydig cells express significantly more GPER in infected animals when comparing to non-infected males (Figure 3).

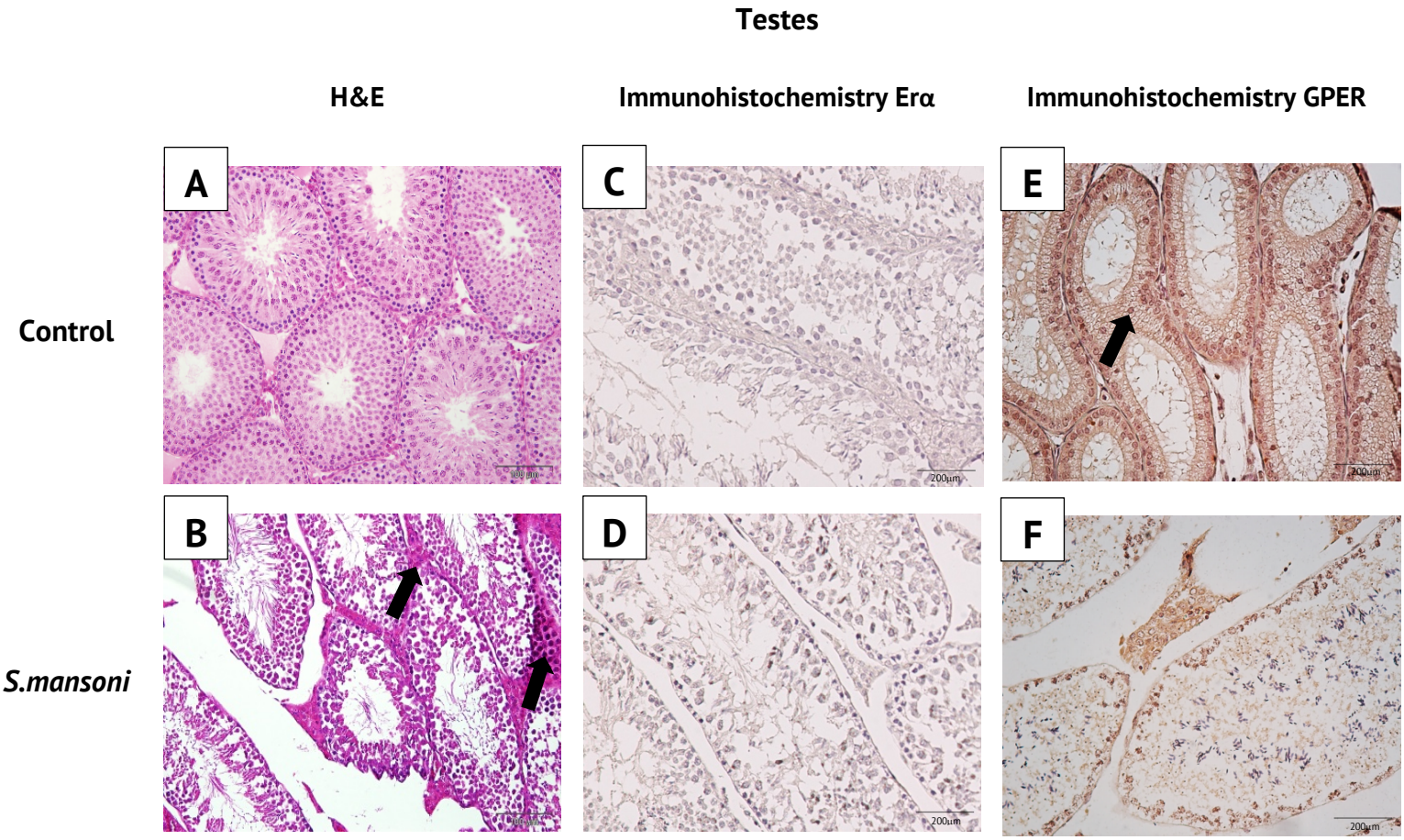

Figure 3.H&E staining and immunohistochemistry (ERα and GPER) of testes in control vs. infected with S. mansoni mice. H&E of (A) Control and (B) infected testes. (B) shows hyperplasic and hyperchromatic Leydig cells (arrows). IHC of ERα in (C) Control and (D) infected testes. (C) ERα expression was almost absent. (D) some spermatogonia nuclei expressed ERα. IHC of GPER in (E) Control and (F) Infected testes, (E) shows more expression of GPER in particular spermatogonia and Sertoli cells' nuclei (arrow). (F) destruction of Sertoli cells, seminiferous tube's cells express significantly less GPER while Leydig cells express more.

Immunohistochemistry

Expression of estrogen receptors in mice gonads infected with soni was evaluated by means of immunohistochemical analysis with bioinformatics imaging quantification tools (Table 2). Through this method estrogen receptors, Erα and GPER were identified in thin sections of both female and male reproductive organs of infefcted mice with S. mansoni.

In the case of the ERα we found a decrease in this receptor expression in the ovary of infected mice and an increase in their testes and uterus compared to controls (Figure 4).

On the other hand, in the case of GPER, we found an increase in the expression of this receptor in the ovary of infected mice and a decrease in their testes and uterus compared to controls (Figure 5).

Table 2. ERα and GPER expression in tissues among non-infected (control) and infected mice with S. mansoni.

Receptor	Control ovary (mean ± SD)	Infected ovary (mean ± SD)	Pvalue	Control uterus (mean ± SD)	Infected uterus (mean ± SD)	Pvalue	Control testes (mean ± SD)	Infected testes (mean ± SD)	Pvalue
ERα	0.0249 ± 0.0044	0.0069 ± 0.0002	0.0285	0.0057 ± 0.0015	0.0362 ± 0.0066	0.0236	0.0070± 0.0064	0.0154± 0.0049	0.0110
GPER	0.0136 ± 0.0159	0.0539 ± 0.0108	0.0015	0.0680 ± 0.0049	0.0382 ± 0.0011	0.0005	0.0922± 0.0158	0.0497± 0.0090	0.0034

ERα

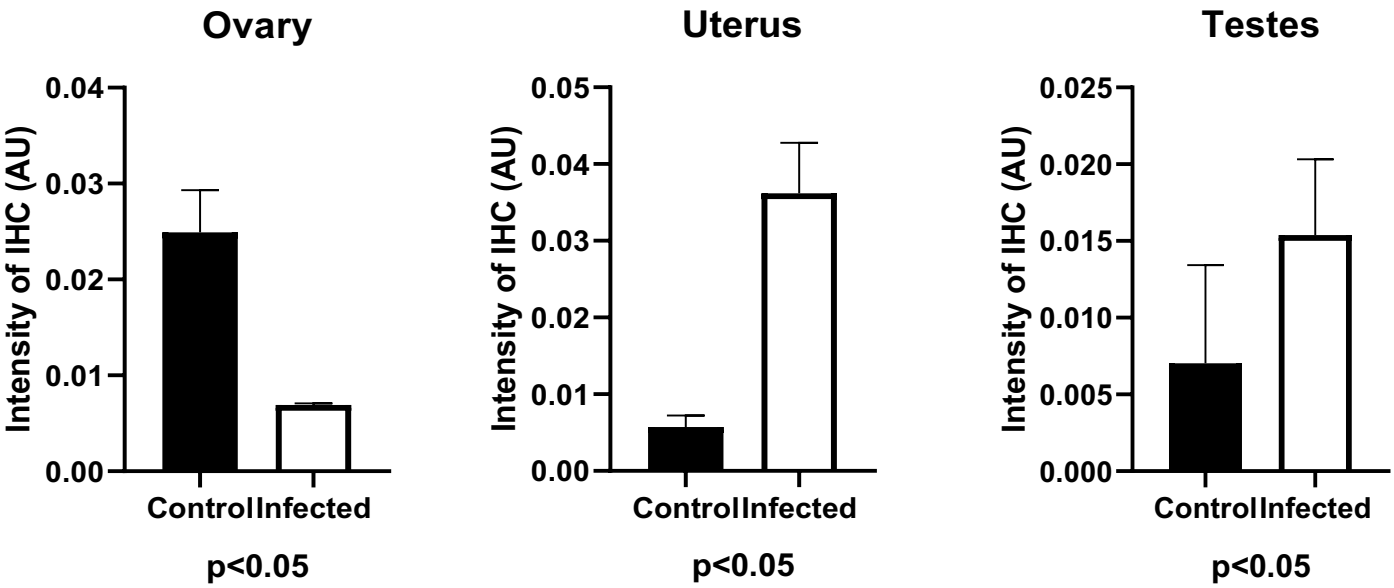

Figure 4. Immunohistochemistry (IHC) results of ERα expression (in arbitrary units, AU) in ovary, uterus and testes of mice.

GPER

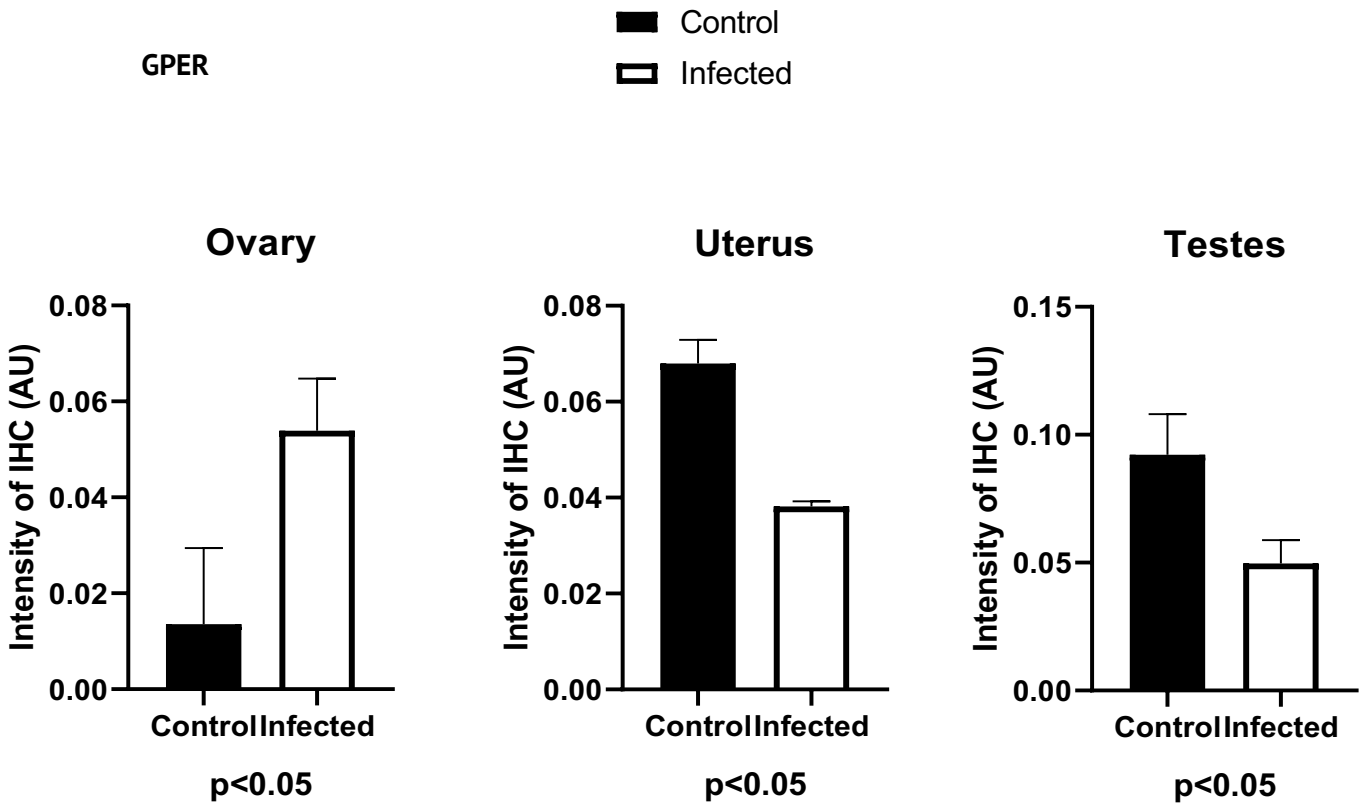

Figure 5. Immunohistochemistry (IHC) results of GPER expression (in arbitrary units, AU) in ovary, uterus and testes of mice.

5. Discussion

There are very few reports associating infertility with S. mansoni infection. The relationship between S.mansoni infection and hormonal alterations is less explored such as in S.haematobium [39].

Estrogen plays a role in many physiological processes and it exerts its effects through three known estrogen receptors: ERα, ERβ and GPER [8].

In the present work, we demonstrated that infection with S. mansoni alters significantly the expression of estrogen receptors, ERα and GPER, both in infected female and male mice.

Estrogens are required for female fertility because they are known to be the primary female sex hormone and are responsible for maturation and maintenance of the vagina and uterus and are also involved in ovarian function, such as maturation of ovarian follicles. Since Schistosoma is responsible for several disorders in the female genital system such as complications during pregnancy and related outcomes, menstrual disorders and other problems related to the female genital system, so the hormonal disturbances in women with FGS may be linked to infertility[12,38,39].

Some genital problems due to S. mansoni have been reported in the form of atrophy of the corpus luteum cells and nuclear alterations of the interstitial cells in the ovaries of mice [38]. Farah et al [9] reported S. mansoni to be a contributing factor in diminished fecundity among women in endemic regions.

It is believed that the presence of parasite-derived catechol-estrogens may influence infertility during infection with Schistosoma through the blockage of estrogen receptors acting as endocrine disruptors [39]. In the present study our group investigated the expression of ERα and was able to demonstrate that in the case of the ovary of S.mansoni infected female mice there is a decrease in the expression of this receptor compared to controls but in uterus there was an increase in expression of this receptor. These results are in agreement with some studies with Selective estrogen receptor modulators (SERM) that show that they act as ER antagonists in the mammary glands and ovary but, on the other hand, act as agonists in the uterus. So this parasite-derived catechol-estrogens may have the ability to inhibit the ERα in some tissues and activate in other [8,51,52].

Some authors already described an increase in the expression level of estrogen receptors in the uterus and endometrium of S. mansoni infected female mice. This phenomenon may happen as a mechanism of compensation due to the decreasing hormonal levels of estrogen and progesterone [39,53].

Estrogens play important roles in the regulation of testis development and spermatogenesis [54,55]. In our study the expression of ERα in testes of infected male mice increased compared to controls. Oliveira et al. [39] found that E2 is below normal levels amplifying infertility observed in

infected females but is augmented in males, explaining hypogonadism and consequent infertility. Previous studies from our group already demonstrated an increase of hormonal levels of Estradiol in S. haematobium and S. mansoni infected male patients and explained that possibly the levels of estradiol accompany the levels of testosterone since estradiol is also synthesized from testosterone. So, studies with human males revealed that increased levels of testosterone (and consequently estradiol) could be a cause of infertility associated with S. mansoni infection in males [39].

Regarding GPER expression, there is no study demonstrating its expression in schistosoma infection. Our group demonstrated that the expression of GPER was the opposite of ER, its expression was increased in the ovaries of infected female mice and decreased in the uterus and testes of both infected female and male mice compared to controls.

Concerning the increased GPER expression on ovary, an endometriosis study has shown that GPER expression is upregulated in the ovarian stroma in cases of endometriosis or septic inflammation. The authors indicated a potential role for GPER in both ovarian physiology and pathophysiology [60]. In the case of the decreased GPER expression in uterus, Wang et al. [61] in a study with patients with polycystic ovary syndrome found that expression of GPR30 was decreased in endometrium of this patients causing lower pregnancy rate and higher spontaneous abortion rates.

GPER has an important role in estrogen signaling regulating the proliferative and apoptotic pathways involved in spermatogenesis throughout rat reproductive development [62].

Functions and cellular composition of the testes change during development and during formation of male infertility but the underlying endocrine mechanisms are unclear [62,63]. As said, in our studie, the expression of GPER were decreased in infected male mice. Sandner et al. [63] examined samples of infertile men with different forms of impaired spermatogenesis and found that in contrast to testicular biopsies from men with normal spermatogenesis showing regular peritubular GPER expression, samples from infertile men exhibited a reduced or even deficient GPER immunostaining.

In spite of substantial evidence that points towards a key role of Schistosomiasis in infertility of infected hosts, current knowledge of the fundamental mechanisms by which this phenomenon happens, is scarce and fragmented. Nonetheless, the relentless progress in ERs and estrogen metabolism investigation and their role in bioprocesses provides us with unprecedented opportunities to thoroughly characterize the functions of such ERs in Schistosomiasis. Here, we contribute to this discussion with our data. The new knowledge can be expected to provide us with a plethora of opportunities to exploit parasite–host associations to our advantage, for example, by applying cutting-edge novel estrogen receptor modulators as innovative intervention strategies against schistosomes and the diseases that they cause.

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