TARGETING MOLECULAR SIGNALING PATHWAYS OF *SCHISTOSOMA HAEMATOBIUM* INFECTION IN BLADDER CANCER

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LISTA DE PUBLICAÇÕES

De acordo com o Artigo 8º do Decreto-Lei nº388/70, fazem parte integrante desta dissertação os seguintes trabalhos publicados ou em publicação:

I - Botelho MC, Oliveira PA, Vieira P, Delgado ML, Lourenço L, Lopes C, Machado JC and Correia da Costa JM. Granulomatous-like immune reaction and hepatic fibrosis induced by *Schistosoma haematobium* immature worms. Virulence 1:3, 2010.

II - Botelho M., Ferreira AC, Oliveira MJ, Domingues A, Machado JC and Correia da Costa JM. *Schistosoma haematobium* total antigen induces increased proliferation, migration and invasion, and decreases apoptosis of normal epithelial cells. Int J Parasitol. 39: 1083-1091, 2009.

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IV - Mónica C Botelho, Paula A Oliveira, Carlos Lopes, José M Correia da Costa and José C Machado. Urothelial dysplasia and inflammation induced by *Schistosoma haematobium* total antigen instillation in mice normal urothelium. Urologic Oncology, doi: 10.1016/j.urolonc.2009.09.017.

V - Mónica C Botelho, Isabel Veiga, Paula A Oliveira, Carlos Lopes, Manuel Teixeira, José M Correia da Costa and José C Machado. Carcinogenic ability of *Schistosoma haematobium* through oncogenic mutation of *KRAS* gene (In Preparation).

VI - Monica C Botelho; Madalena Crespo; André Almeida; Paulo Vieira, Maria L Delgado, Luís Araújo; José C Machado; José M Correia da Costa. *Schistosoma haematobium* and *S. mansoni*: production of an estradiol-related compound detected by ELISA. Exp Parasitol. 122: 250-253, 2009.

VII - Botelho MC, Soares R, Vale N, Ribeiro R, Camilo V, Almeida R, Medeiros R, Gomes P, Machado JC, da Costa JM. *Schistosoma haematobium*: Identification of new estrogenic molecules with estradiol antagonistic activity and ability to inactivate Estrogen Receptor in mammalian cells. Exp Parasitol. 126: 526-535, 2010.

VIII - Monica Catarina Botelho, Jose Carlos Machado and Jose Manuel Correia da Costa. *Schistosoma haematobium* and bladder cancer: What lies beneath? Virulence 1:2, 2010.

IX - Mónica Catarina Botelho, Ricardo Ribeiro, Nuno Vale, Paula Oliveira, Rui Medeiros, Carlos Lopes, José Manuel Correia da Costa and José Carlos Machado. Inactivation of Estrogen Receptor by *Schistosoma haematobium* total antigen in bladder urothelial cells (In Preparation).

Em cumprimento do disposto no referido Decreto-Lei, declara que participou activamente na recolha e análise de dados e escreveu todos os artigos com a colaboração dos co-autores.

NOTA EXPLICATIVA

A presente dissertação encontra-se escrita em Inglês na sua quase totalidade devido ao facto do Prof. Paul J. Brindley, da Universidade de Washington, ser um dos arguentes.

Para a minha família

“The noblest pleasure is the joy of understanding. “

Leonardo da Vinci

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LIST OF ABBREVIATIONS

AF – Activation function

BCA - bicinchoninic acid

Bcl-2 - B-cell lymphoma 2

BD – Becton Dickinson

C16 – Carbon 16

C17 – Carbon 17

C2 – Carbon 2

C4 – Carbon 4

CD-1 – Cluster of differentiation 1

CDKI - cyclin-dependent kinase inhibitor

cDNA – Complementary Deoxyribonucleic acid

CHO – Chinese hamster ovary

CIBP – Centro de Imunologia e Biologia Parasitária

CO_2 – Carbon dioxide

DALYs - disability-adjusted life years

DNA - Deoxyribonucleic acid

E2 - estradiol

ECLIA - Electrochemiluminescence Immunoassay

ELISA - Enzyme Linked Immuno Sorbent Assay

ER – Estrogen receptor

ERE – Estrogen responsive element

ES – excretion/secretion

ESI - electrospray ionization

ESR1 – Estrogen receptor 1 gene

ESR2 - Estrogen receptor 2 gene

FACS - Fluorescence Activated Cell Sorting

FBS - Fetal bovine serum

GADPH - Glyceraldehyde 3-phosphate dehydrogenase

GPR30 - G-protein coupled receptor 30

H&E – Hematoxylin and eosin

HIV/AIDS – Human immunodeficiency virus / Acquired immune deficiency syndrome

HPLC - High-performance liquid chromatography

HRP - horseradish peroxidase

IARC – International agency for research on cancer

ICI – ICI 182,780

IPATIMUP – Institute for pathology and molecular immunology of porto university

KOH - Potassium hydroxide

KRAS - Kirsten rat sarcoma viral oncogene homolog

LC – Liquid chromatography

LC-ESI-MS - Liquid chromatography−electrospray ionization−mass spectrometry

LC-MS - Liquid chromatography-mass spectrometry

LGIUN – Low grade intraurothelial neoplasia

LH - Luteinizing Hormone

mRNA - messenger ribonucleic acid

MS – Mass spectrometry

MTS-(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

NO – nitric oxide

PBS - Phosphate buffered saline

PCR – Polymerase chain reaction

PI - Propidium iodide

PMS - phenazine methosulfate

Ras – Rat sarcoma

RNA - ribonucleic acid

RPMI - Roswell Park Memorial Institute medium

RT-PCR – Real time polymerase chain reaction

SCC – Squamous cell carcinoma

SD – Standard deviation

Sh – *Schistosoma haematobium* total antigen

SMC – smooth muscle cells

S-phase – synthesis phase

SPSS - Statistical Package for Social Sciences

SWAP – Schistosome worm antigen preparation

TCC – Trasitional cell carcinoma

Th2 – T helper 2

TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling

USA – United States of America

UV – Ultraviolet

WHO – World health organization

BACKGROUND

SCHISTOSOMIASIS: GENERAL ASPECTS

Anthropophylic *Schistosoma* species currently infect more than 200 million people in 76 countries worldwide, according to the World Health Organization, in the endemic areas of Africa, the Caribbean, Central America, South America, East Asia, and the Middle East. Prevalence is thought to be rising mainly due to increasing frequent travelers from USA and Europe to these endemic regions for business or leisure. Mortality in human schistosomiasis is thought to be around 500 thousand every year and is related to complications of fibro-occlusive disease secondary to the immune stimulus of Schistosoma ova and end organ damage (Herrera et al, 2005).

Most broad generalizations about schistosomiasis state, first, that it is a rural disease and, second, that it affects the poor populations. The changing global demography requires that the first assumption be reconsidered. Urban schistosomiasis is a reality rather than a potential threat. Urban schistosomiasis is the cumulative outcome of local transmission and migration (Ken Mott, 1989). Movements of displaced person due to conflict, drought and famine are currently playing an important role in the changing epidemiology of schistosomiasis in these countries: 1) Laos/Cambodia/Thailand; 2) Ethiopia/Somalia/Sudan; 3) Mozambique/Zambia/Malawi; Angola/Zaire (Ken Mott, 1989).

Schistosomiasis was included in the list of neglected tropical diseases. However, a new era must come, illuminated by the experience and work of so many scientists all over the world. For two main challenges: 1) toward a better comprehension of the pathological and effectors of immunological mechanisms; 2) to promote a better life to the people living in endemic regions, because schistosomiasis is a real barrier to their social and economic development. A significant signal appears in July 2009: the complete genomic sequencing of *Schistosoma mansoni* and *Schistosoma japonicum* was published in Nature. New evidences are coming

(Berriman et al, 2009; Schistosoma *schistosoma* Genome Sequencing and Functional Analysis Consortium, 2009)

SCHISTOSOMIASIS LIFE CYCLE AND INFECTION

The life cycle of human schistosomes begins once cercariae have entered the body. They are called schistosomulae, which migrate through the tissues to invade blood vessels. The organisms are transported to the lungs and then to the liver, where they mature into an adult worm within 6 weeks before descending to their final positions in the venous circulation. Adults of *S haematobium* are mostly found in the venous plexi of the bladder, prostate, and uterus, while *S intercalatum, S japonicum, S mansoni,* and *S mekongi* are observed in the portal, inferior, and superior mesenteric veins (Figure 1). Maturity of female worms depends on the presence of a mature male because they form pairs, with the female lying enclosed within a groove formed by the male (Butterworth et al, 1988; Ouma et al, 2001).

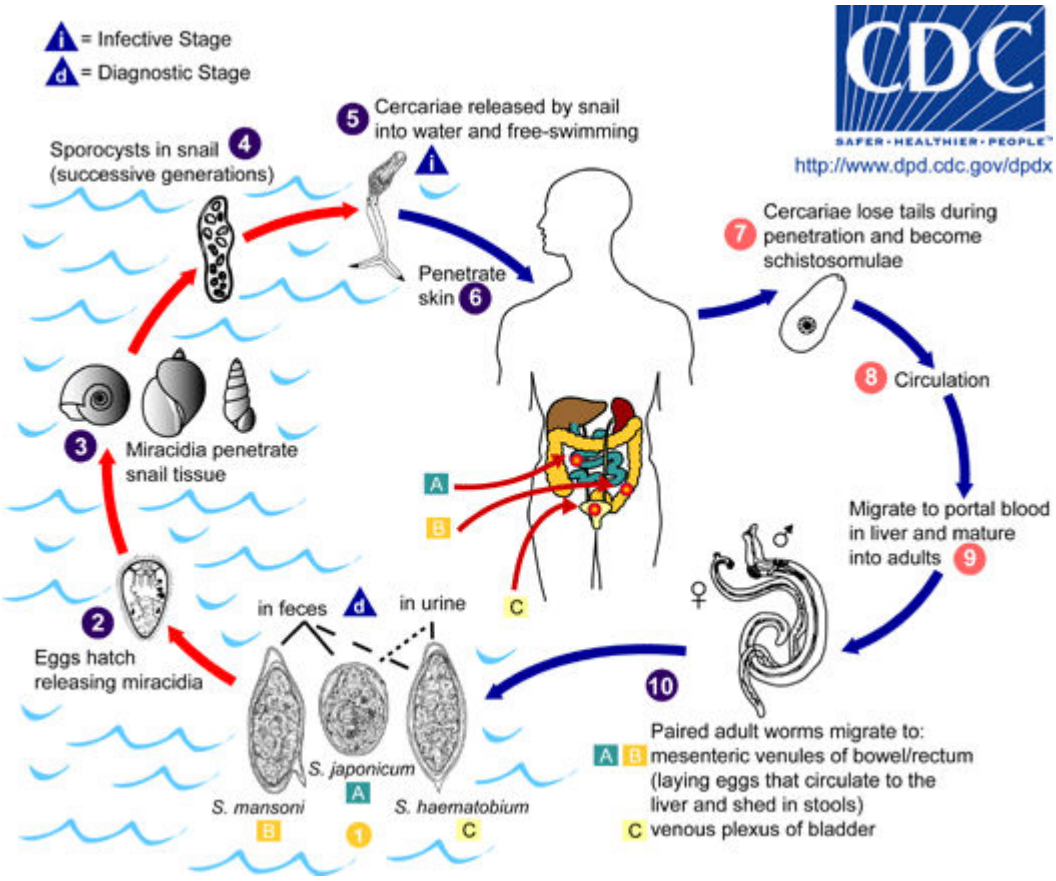


Figure 1: Schistosomiasis life-cycle.

Adult worms have a mean life span of 5-10 years, with females releasing 300-3000 eggs per day. Mature parasites can live in human tissues for more than 30 years (Vieira et al, 2007). Eggs are deposited in the terminal venules of the bladder (*S haematobium*), intestine, and rectum (*S intercalatum, S japonicum, S mansoni, S mekongi*), where they mature to contain a miracidium over the next 10 days. The schistosomulae releases proteolytic enzymes, which facilitate larval movement through the tissues into either the genitourinary tract or the gastrointestinal tract. Eggs are passed in human urine and excrement, and motile miracidia are released upon contact with fresh water. Miracidia then actively seek out snail hosts in which they develop first into mother and daughter sporocysts by asexual division and then into cercariae over 4-6 weeks. Cercariae then leave the snail and penetrate the human skin on contact with the assistance of their glandular secretions (Butterworth et al, 1988; Ouma et al, 2001).

Mortality from human schistosomiasis is related to complications of fibro-occlusive disease secondary to the immune stimulus of schistosome eggs and end organ damage (Herrera et al, 2005). Mortality is thought to be around several thousands every year. There is a great lack of information in the real numbers, mainly because the countries where prevalence is higher have insufficient or inexistent registries.

Clinical features of human schistosomiasis depend on the species, developmental stage, and site of infection in the body. This can be summarized into 3 major syndromes: (1) cercarial dermatitis, (2) acute schistosomiasis or Katayama fever, and (3) chronic fibro-obstructive disease (Butterworth et al, 1988; Herrera et al, 2005; Sturrock et al, 2001).

SCHISTOSOMIAIS: ASPECTS OF EPIDEMIOLOGY

Schistosomiasis is an ancient scourge of mankind, depicted graphically in papyri from Pharaonic Egypt and known from human remains over 2000 years old from China (Adamson, 1976). Human schistosomiasis is caused by 1 of 6 species: *Schistosoma haematobium*, *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma intercalatum*, *Schistosoma malayensis* and *Schistosoma mekongi*. Schistosomiasis was originally called bilharzia in homage to Theodor Bilharz, a young German pathologist who first described the disease and its association with *Schistosoma*, working at Cairo in Egypt. Blood-dwelling Trematoda (phylum Platyhelmintha) of the genus *Schistosoma* cause this chronic and debilitating disease (Steinmann, 2006). In a different manner of other flatworms, *Schistosoma* are digenetic and elongated, becoming round-shape as an adaptation manner to residing in blood vessels of the genitourinary or gastrointestinal tracts. They present a heteroxenic life cycle, requiring an invertebrate as secondary host, water-dwelling snails of genus *Bulinus, Oncomelania*, or *Biomphalaria*. Geographic distribution and maintenance of human infection by *Schistosoma* is dependent on and limited by the presence of a suitable snail host.

Schistosoma adult worms are sexually distinct, (a characteristic that separates them from other flukes that are hermaphrodites). However, male present a ventral infolding from the ventral sucker to the posterior end forming the gynecophoric canal, where female are kept during copulation (copulatory groove) (Figure 2). *Schistosoma* life cycle differs from that of other flatworms also by the infecting process of the definitive host: the penetration of cercariae through the skin (per cutem) rather than through oral ingestion (per os). Human *Schistosoma* can infect other vertebrates and provide an animal reservoir of infection, although it is of epidemiologic significance only for *S. japonicum* and possibly *S. mekongi*. Snails of the genus *Biomphalaria, Bulinus, Neotricula*, and *Oncomelania* are the principle intermediate hosts for *S. mansoni, S. haematobium, S. mekongi,* and *S. japonicum*, respectively (Butterworth et al., 1988).

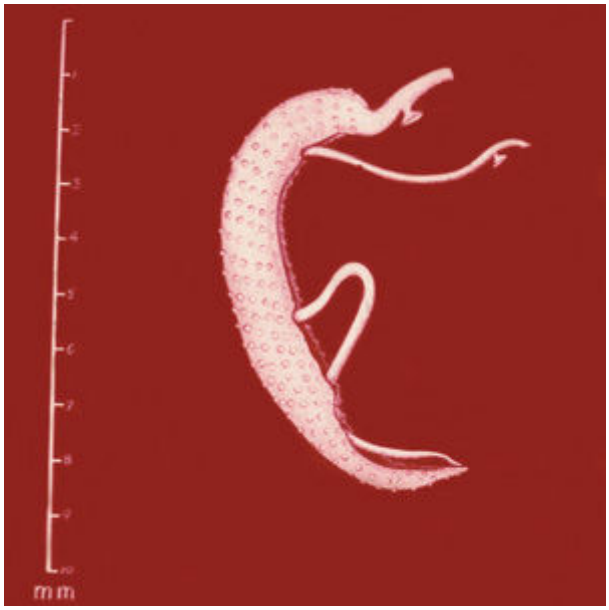


Figure 2: Adults of S. *mansoni*. The thin female resides in the gynecophoral canal of the thicker male. Note the tuberculate exterior of the male (DPDx, Schistosomiaisis Image Library, Centers for Disease Control and Prevention).

Human schistosomiasis infects people in the endemic areas of Africa, the Caribbean, Central America, South America, East Asia, and the Middle East. Prevalence is thought to be increasing. The most severely affected countries in Africa are Angola, Chad, Congo, Egypt, Ghana, Kenya, Madagascar, Malawi, Mali, Mozambique, Nigeria, Senegal, Sudan, Tanzania, Uganda, Zambia, and Zimbabwe. Yemen has the most infected people in the Middle East. Brazil is the most affected country in the Americas, with 25 million people living in endemic areas and an estimated 3 million infected (Herrera et al, 2005).

S. haematobium is endemic in 53 countries in the Middle East and most of the African continent, including the islands of Madagascar and Mauritius. The infection is unlikely to be of public health significance in Lebanon, Oman, Syria, Tunisia, and Turkey because transmission is low or nonexistent. A disputed and ill-defined focus exists in India and requires further confirmation (Steinmann et al., 2006).

S. mansoni is found in 54 countries, including the Arabian Peninsula, Egypt, Libya, Mauritania, Somalia, Sudan, sub-Saharan Africa, Brazil, the Caribbean (except Antigua, Guadeloupe, Martinique, Montserrat, and St. Lucia), Suriname, and Venezuela (Steinmann et al., 2006).

S. japonicum is endemic in China, the Sulawesi province of Indonesia, and the Philippines. China is the most affected country, with an estimated 900 000 people infected. The parasite has been eradicated from Japan since 1982 (Steinmann et al., 2006).

S. intercalatum has been reported from 10 countries in central and western Africa. *S. mekongi* is confined to Cambodia and Laos where the borders run along the Mekong River. *Neotricula* snails have been reported in southern China, but no reports of *S. mekongi* exist from these areas. *S. malayensis,* and its intermediate host *Robertsiella* snails, were found in a small jungle focus in Malaysia infecting aboriginal people (WHO).

According to Wright (1972) schistosomiasis was recognized to be endemic in 71 countries although neither India nor Mauritius was included among these. Before that time transmission of schistosomiasis had ceased in Cyprus and Portugal. Cyprus and Portugal were known, during the first half of the last century, as the only autochthonic endemic focus of

schistosomiasis in Europe. Since that time no new or old infections were declared in St. Martin and Israel and are no longer included. Since 1972, additional countries reporting schistosomiasis are Equatorial Guinea, India, Jordan, Malaysia, Mauritius. Montserrat, Oman and São Tomé e Príncipe. As of 1989 schistosomiasis is endemic in 76 countries (Ken Mott, 1989).

Before 1972 transmission of schistosomiasis had ceased in Portugal. Nowadays, schistosomiasis is not endemic in Portugal. However, between 1961 and 1974, the country was involved in African colonial wars, which led to the dispatching of Portuguese military personnel to Angola, Mozambique and Guinea-Bissau, all with endemic *S. mansoni* and *S. haematobium*. Upon the return of military personnel to Portugal, medical services were provided. In the only report in 1971, stools and urine from 3206 of the returnees were examined microscopically, and 3, 4% were found to be positive for S. *schistosoma* and 2, 8% to be positive for S. haematobium. This was evidence that Portuguese repatriates had been exposed to parasites, and more would probably have been found to be infected had they been examined by trained personnel (Coutinho da Costa, 1971).

HELMINTHIASES AS NEGLECTED TROPICAL DISEASES

Parasitic worms from the phyla Plathelmintha (flatworms) and Nematoda (roundworms), all together as helminth parasites, they comprise the most common infectious agents of humans in developing countries, estimated as one-third of the Humanity. Based on a recent analysis by Hotez and collaborators (2009) 85% of the neglected tropical disease burden for the poorest 500 million people living in sub-Saharan Africa results from helminth infections. Hookworm infection occurs in almost half of the poorest people in this part of Africa, including 40–50 million school-aged children and 7 million pregnant women, in whom it is a leading cause of anaemia. Schistosomiasis (192 million cases) is the second most prevalent neglected tropical disease after hookworm, accounting for 93% of the world's number of cases of schistosomiasis and possibly associated with increased horizontal transmission of HIV/AIDS. Lymphatic filariasis

(46–51 million cases) and onchocerciasis (37 million cases) are also widespread in Africa, and each disease representing a significant cause of disability and reduction in the region's agricultural productivity. The disease burden estimate in disability-adjusted life years (DALYs) for total helminth infections in sub-Saharan Africa is 5.4–18.3 million in comparison to 40.9 million DALYs for malaria and 9.3 million DALYs for tuberculosis. Research into helminth infections has not received nearly the same level of support. This is partly because helminthiases are diseases of the poorest people in the poorest regions, but also because these pathogens are difficult to study in the laboratory by comparison to most model eukaryotes and many other pathogens. Standard tools and approaches, including cell lines, culture *in vitro* and animal models, are generally lacking (revised by Brindley et al., 2009 and Hotez et al., 2009). Helminth diseases are ancient scourges of humanity, with some known from biblical times. However, most can also be considered as re-emerging diseases in the sense that new outbreaks are reported routinely in response to environmental and socio-political changes. Schistosomiasis has re-emerged many times in Africa in recent times in response to hydrological changes, e.g. construction of dams, irrigation canals, reservoirs, etc. that establish suitable new environments for the intermediate host snails that transmit the parasites. Schistosomiasis has also re-emerged in mountainous and hilly regions in Sichuan, China, where it had been controlled previously by intensive interventions. Furthermore, new strains of Schistosoma are indeed emerging through natural hybridizations between human and cattle species of Schistosoma. What's more, research on immunology of helminth infections has contributed enormously to our understanding of Th2 immune responses, the function of regulatory T cells, generation of alternatively activated macrophages, and the transmission dynamics of infectious agents? It is hoped that this progress can be translated into new drugs, diagnostics, and vaccines for the helminth diseases (revised by Patz JA et al., 2000; Liang S et al., 2006; Huyse T et al., 2009; Brindley PJ et al., 2009).

ASPECTS OF THE MOLECULAR BIOLOGY OF SCHISTOSOMA.

Brindley (2005) reviewed the molecular biology of *Schistosoma*. According to the molecular analyses of ribosomal RNA genes and mitochondrial genes, *Schistosoma* worms probably originated in Asia and were subsequently dispersed through India and Africa. Also, the analyze of nuclear genome sequences of *Schistosoma* indicates that worms encode mammalian-like receptors for insulin, progesterone, cytokines and neuropeptides, suggesting that host hormones, or endogenous parasite homologues, might orchestrate parasite development and maturation, and that worms modulate antiparasite immune responses through inhibitors, molecular mimicry and other evasion strategies.

These blood flatworms are able to utilize the amino acids from host haemoglobin for growth, development and reproduction. Genes encoding the haemoglobin-degrading hydrolases, namely cathepsins or leucine aminopeptidase's, have been characterized. Also, they use serum lipoproteins, blood-group glycolipids, histocompatibility antigens and other host molecules to circumvent deficits in their metabolic pathways or to deflect immune recognition. However, they cannot synthesize cholesterol or fatty acids *de novo* and seem to obtain these nutrients from the host (Brindley, 2005).

Finally, Berriman and collaboratores (2009) present analysis of the 363 megabase nuclear genome of the blood fluke *Schistosoma mansoni*. It encodes at least 11,809 genes, with an unusual intron size distribution, and new families of micro-exon genes that undergo frequent alternative splicing. As the first sequenced flatworm, it offers insights into early events in the evolution of the animals, including the development of a body pattern with bilateral symmetry, and the development of tissues into organs. They remark deficits in lipid metabolism that make *Schistosoma* worms dependent on the host, and the identification of membrane receptors, ion channels and more than 300 proteases provide new insights into the biology of the life cycle and new targets.

SCHISTOSOMA HAEMATOBIUM AND ITS ASSOCIATION TO SQUAMOUS CELL CARCINOMA OF THE BLADDER

Not all schistosome eggs are excreted from the body, and up to 50% can embolize to other body areas, leading to a host immune reaction and granuloma formation. Granulomas begin to form with maturation of the miracidium at 6 days and are focal within 2 weeks. The most common sites are the liver for *S. intercalatum, S japonicum*, and *S mansoni* and the bladder for *S haematobium*. Other areas less commonly affected include the lungs, central nervous system, and kidneys. During infection by *S haematobium*, eggs are deposited in the mucosa and submucosa of the bladder and lower ureters. Granulomas are very cellular and form intraluminal polyploidal lesions that can lead to hydronephrosis (Figure 3). Lesions tend to necrotize, ulcerate, and bleed. With age, the lesions become acellular, fibrotic, and calcify and are termed sandy patches. Calcification may lead to bladder deformation, ureteric obstruction, secondary infections, hydronephrosis, chronic pyelonephritis, and renal failure. Carcinoma of the bladder is a long-term sequela of chronic infection (Capron et al, 2005; Hoder et al, 2000; Pierrot et al, 2001; Trottein et al, 2004).

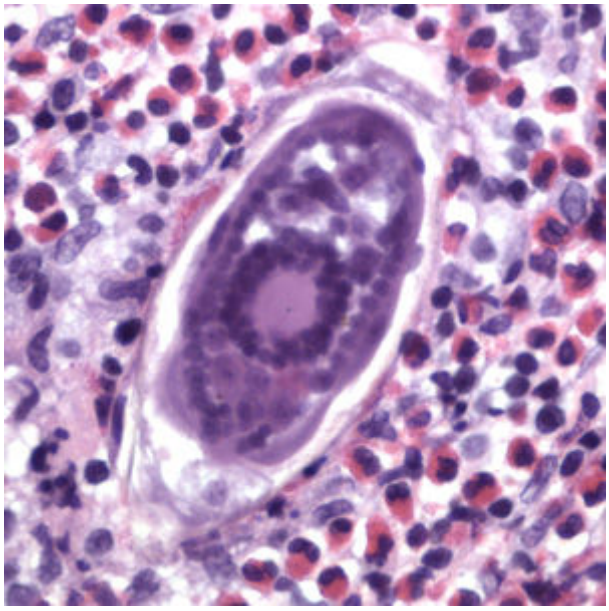


Figure 3: Eggs of S. haematobium in a urinary bladder biopsy specimen, stained with H&E (Image courtesy of the Michael E. DeBakey V. A. Medical Center, Houston, TX, DPDx, Schistosomiaisis Image Library, Centers for Disease Control and Prevention).

Bladder cancer is one of the most severe complications of chronic schistosomiasis. Case report studies indicate that individuals with schistosomiasis may develop bladder cancer earlier than uninfected people. The severity and frequency of the sequela or urinary schistosomiasis and of its complications (urothelial cancers) depend on the intensity of infection (worm burden and tissue egg burden, and the duration of infection (Herrera et al, 2005; Hoder et al, 2000).

Squamous cell carcinoma of the urinary bladder has been associated with *Schistosoma haematobium* infection in many parts of Africa. The epidemiologic association is based both on case-control studies and on the close correlation of bladder cancer incidence with prevalence of *S. haematobium* infection within different geographic areas. It has been estimated a Schistosoma-associated bladder cancer incidence of 3-4 cases per 100.000 (Shiff et al., 2006). A parasite-tumour linkage is further suggested by the predominance of squamous cell (as opposed to transitional cell) morphology of bladder carcinomas seen in S. haematobium-

endemic areas, and by the frequent association of tumours with parasite ova and egg-induced granulomatous pathology in involved bladder tissues.

Another factor that may play a major role in bladder carcinogenesis in Schistosomiasis patients is the presence of continuous physical irritation and inflammation produced by *schistosoma*l eggs in the bladder mucosa. The adult *S. heamatobium* worms inhabit the veins of the perivesical plexus, where the female lays eggs. Some eggs pass through the bladder mucosa and are excreted in the urine. Other eggs are trapped in the tissue. A chronic inflammatory reaction is initiated, with the invasion of histiocytes and other inflammatory cells into the bladder, the formation of granulomas, and eventually fibrosis. In addition, the eruption of the eggs through the mucosa stimulates reparative urothelial hyperplasia and cell turnover (Rosin et al, 1994). Moreover, in association with schistosomiasis, cancer may originate throughout the bladder but rarely in the trigone, a frequent site of tumour origin in non-*schistosoma*l cases. The tumours are often first seen in an advantage stage, arising from the posterior bladder wall and vault. The trigone is only affected in 8.5% of the cases. The reason accounts for the striking deformities in the urethers, including medial deviation, a straight lumbar course, and in the pelvic segment a bowed appearance with medial and cranial displacement at the vesico-ureteric junction (Tawfik, 1987). Stasis and dilatation of the upper urinary tract are present in the absence of mechanical obstruction and these changes in the upper urinary tract are due to functional derangement of the urethers (Umerah, 1977).

Human bladder cancer is the fifth to the seventh most common cancer in Western countries. In many tropical and subtropical areas, however, it is the first among all types of cancer, mainly due to the endemic parasitism (Badawi, 1996). Felix et al (2008) has shown that the occurrence of TCC (transitional cell carcinoma) of the bladder has supplanted the SCC (squamous cell carcinoma) in Egypt following a major decline in the prevalence of urinary schistosomiasis. Such decline in the pattern of this infection suggests the importance of Schistosoma-associated bladder cancer that, elsewhere, may be more widespread than is presently thought (Shiff, 2010).

The mechanisms underlying the association between *S. haematobium* and squamous cell carcinoma of the bladder (SCC) are largely unknown. Firm understanding of the

pathobiologic features of this disease is necessary to understand their role in the direction of treatment.

S. HAEMATOBIUM INFECTION AND THE HOST ENDOCRINE SYSTEM

Hormones regulate a variety of cellular and physiological functions of organisms, such as growth, reproduction and differentiation. The mechanisms by which host hormones act on parasites have recently been investigated, and some parasite molecules that are involved in transregulation have been identified and characterized (Escobedo et al, 2005).

In the case of schistosomes, hormonal signals from the host seem to have a major influence on larval homing, survival, growth and sexual maturation. Moreover, this is a two-way dialogue and helminth parasites have a particularly marked effect on the fecundity of the host (Mendonça et al, 2000).

Nuclear hormone receptors comprise a large superfamily of transcription factors whose activity is under hormonal control. These receptors are characterized by a central DNA-binding domain, which interacts with specific hormone response elements located near the target gene promoter, and by two distinct activation function (AF) domains that contribute to the transcriptional activity of these receptors (Delage-Mourroux et al, 2000).

The estrogen receptor (ER), a member of the steroid receptor family, mediates the stimulatory effects of estrogens and the inhibitory effects of antiestrogens in estrogen target cells (Martini P et al, 2000). ER mediates the biological effects of estrogens in a variety of target tissues (Delage-Mourroux et al, 2000) and ER-regulated genes are involved in many biological processes, including cell growth and differentiation, morphogenesis, and programmed cell death (Martini P et al, 2000). The ligand binding to estrogen receptor stimulates gene transcription via interaction with the estrogen response element (ERE) (Catherino and Jordan, 1995).

Estrogens receptors are known to mediate important physiologic functions, including reproduction, metabolism, maintenance of bone density, and growth of estrogen-responsive

tumors, including breast and endometrial cancers. There are 2 nuclear receptors, ER alpha and ER beta, which mediate the physiologic response to estrogen. They share several functionally conserved structural domains such as the regions responsible for DNA binding, dimerization, ligand binding and ligand-dependent transactivation of gene expression (Shen SS et al, 2006). ER alpha and ER beta have different transcriptional activities in certain ligand, cell-type, and promoter contexts and also exhibit distinct tissue- and cell-type specific expression patterns. ER alpha is found predominantly in the normal pituitary, breast, uterus, vagina, testis, liver, and kidney, whereas ER beta is mainly expressed in thyroid, ovary, prostate, skin, bladder, lungs, gastrointestinal tract, bone and cartilage (Shen SS et al, 2006). ER was found to localize to the urethral epithelium but has been reported to be absent from transitional epithelium (Tincello DG et al, 2009).

The knowledge that schistosomes have estradiol receptors as long been attained (Barrabes et al, 1986). This molecule able to bind estradiol has been previously described in schistosomes. It could be used by the parasite, as a likely mechanism for the protective effect of this hormone in infected mice and hamsters (Barrabes et al, 1986).

Recent experimental evidence suggests that schistosomes can not only evade immune responses actively**,** but also exploit the hormonal microenvironment within the host to favor their establishment, growth**,** and reproduction (Escobedo et al**.**, 2005). It is generally believed that infection by schistosomes' leads to modifications in the hormonal status of their hosts and that this is the result of a secondary reaction of the host to infection, one that is not directly mediated by parasite-derived factors (Mendonça et al**.**, 2000). It was therefore hypothesized that schistosomes produce hormones and that this hormones interact with hormonal receptors in the host.

AIMS AND STUDY OUTLINE

The general objective of this study was to investigate the association between Schistosoma haematobium infection and bladder squamous cell cancer in endemic areas of vesical schistosomiasis. With this in mind, the overall aims of this work were: a) to study the mechanisms underlying the tumorigenic effects of S. haematobium infection in squamous cell carcinoma (SCC) of the bladder; and b) to understand how the host endocrine system can favor the establishment of schistosomes. To achieve these goals we followed the study outline explained below:

A) TO STUDY THE MECHANISMS UNDERLYING THE ASSOCIATION BETWEEN S. HAEMATOBIUM AND SCC OF THE BLADDER

I. TO STUDY THE EFFECTS OF *S. HAEMATOBIUM* TOTAL ANTIGEN IN NORMAL MAMMALIAN CELLS AND TO INVESTIGATE ITS INVOLVEMENT IN TUMOURIGENESIS

First we obtained evidence that immature male worms of *S. haematobium* alone caused hepatic lesions in the golden hamster, similar to those described for periovular granuloma. This study provided further experimental evidence for the role that schistosome worms, and their derived antigens, may play in the pathology of the infection of schistosomiasis, and prompted us to study the effect of *S. haematobium* total antigen in normal cells.

In our second paper we hypothesized that the parasite antigens might induce alterations in normal epithelial cells towards cancer. For this we used Chinese Hamster Ovary (CHO) cells and treated the cells in culture with *S. haematobium* total antigen (Sh).

To investigate the involvement of Sh in tumourigenesis we used the previous CHO cells exposed to Sh, after which they were inoculated subcutaneously into male nude mice.

II. TO ANALYZE POSSIBLE ALTERATIONS AND SUBSEQUENT MUTATIONS IN UROTHELIUM OF CD-1 MICE INDUCED BY THE PARASITE ANTIGENS.

Previous results encouraged us to investigate the effects of *S. haematobium* total antigen in CD-1 mice normal bladders, after intravesical instillation of the parasite antigens.

To further study specifically the carcinogenic alterations caused by Sh in the bladders of CD-1 mice, we looked for gene mutations in these bladders. Carcinoma of the bladder frequently harbours gene mutations that constitutively activate the receptor tyrosine kinase-Ras pathway and therefore we studied activating mutations in *KRAS* gene.

B) TO UNDERSTAND HOW HOST ENDOCRINE SYSTEM CAN FAVOR THE ESTABLISHMENT OF SCHISTOSOMES

I. TO ADDRESS SCHISTOSOMIASIS-INDUCED HORMONAL IMBALANCES.

While addressing schistosomiasis-induced hypogonadism by several hormones in patients infected with *S. haematobium* and *S. mansoni* (anedoctal cases described by Capron, personal communication), we observed an extraordinary increase in serum levels of estradiol (E2).

II. TO UNDERSTAND THE ROLE OF ESTROGENS PRODUCED BY SCHISTOSOMES.

After identifying the expression of an E2-related molecule by *Schistosoma haematobium* total antigen (Sh), we tested the ability of this estrogenic molecule produced by *S. haematobium* to interact *in vitro* with Estrogen Receptor (ER) in the estrogenic responsive MCF7 cell line.

Since we observed the repression of ER in MCF7 cells by Sh, we tested the hypothesis that Sh would also have the same effect in urothelial cells. Therefore we used HCV29 cells, a cell line of normal urothelial cells, and CD-1 mice to evaluate the expression of ER in the bladders of Sh instilled animals.

MATERIAL AND METHODS

A) MECHANISMS UNDERLYING THE ASSOCIATION BETWEEN S. HAEMATOBIUM AND SQUAMOUS CELL CARCINOMA (SCC) OF THE BLADDER

ANIMALS

Eight-week-old female golden hamsters (LVG/SYR) and CD-1 mice were provided by Charles River (Barcelona, Spain). Male N:NIH(s)II-nu/nu mice of 4-6 weeks old were obtained from IPATIMUP.

Animals spent one week being acclimated under routine laboratory conditions before starting the experiments. They did not receive any treatment prior to the study. Hamsters were kept in separated cages and CD-1 mice were kept in 6 litter mates cage. 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 ± 2ºC) and humidity (55% ± 10%) and continuous air renovation. Nude mice were maintained under sterile conditions throughout the experiment (temperature 24±2ºC, relative humidity 55±5% and a 12 hour photoperiod) in polycarbonate cages. They were fed sterilized autoclave rodent feed and water *ad libitum*. 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).

PARASITES

S. haematobium (Angolan strain) life cycle was maintained through successive passages in laboratory- raised *Bulinus truncatus* as invertebrate hosts and golden hamsters as vertebrate hosts. Cercariae of *S. haematobium* were obtained from infected snails by the use of artificial light.

EXPERIMENTAL INFECTION

Ten hamsters were divided into 4 groups. Groups 1 and 3 (n=2) were control groups. Groups 2 (n=3) and 4 (n=3) were infected with *S. haematobium*. Groups 1 and 2 were sacrificed 26 weeks after infection; group 3 and 4 were sacrificed 82 weeks after infection. Hamsters were experimentally infected by transcutaneous route with approximately 100 cercariae of *S. haematobium*. For the duration of the study, the hamster's state of health was monitored daily.

The immature worm burden was determined by total blood perfusion and the numbers of worms determined. Immature worms of S. haematobium were recovered at 26 and 82 weeks after challenge by perfusion of the hepatic portal system via the aorta with citrated saline. Hamsters were perfused under anesthesia through the heart with citrated phosphate-buffered saline and the worms were recovered. Worm burdens were estimated after portal perfusion through an incised portal vein of infected mice euthanized by an anesthetic overdose.

At the time of perfusion, complete necropsies were carefully conducted. After opening the abdomen, all organs were examined macroscopically for any changes; the organs were collected, weighed and immersed in 10% phosphate buffered formalin. Representative fragments of all organs were fixed in buffered formalin 10%. Tissue sections (2 µm) were stained with each of the following: haematoxylin and eosin (HE); Wright for eosinophiles and reticulin and Masson trichrome for collagen.

S. HAEMATOBIUM TOTAL ANTIGEN PRODUCTION

Angolan strain of *S. haematobium* adult worms was collected by perfusion of the hepatic portal system of golden hamsters at seven weeks after infection with 100 cercarie. The worms were suspended in PBS and then sonicated. The protein extract was then ultracentrifuged and the protein concentration was estimated using a micro BCA protein assay reagent kit (Viana da Costa A et al, 1998).

CELL LINES

CHO (Chinese Hamster Ovary) cells were cultured and maintained at 37º C in a 5% CO_2 humidified atmosphere in CHO medium (Sigma) with 10% FBS and 1% penicillin/streptomycin (Sigma). Cells were passaged every 5 days. Before treatments cells were serum-starved for 16 h.

PROLIFERATION ASSAY

The CellTiter 96 AQ nonradioactive cell-proliferation assay (Promega) was used to assess cell viability. The assay is composed of the tetrazolium compound MTS and an electron coupling reagent, PMS. MTS is reduced by viable cells to formazan, which can be measured with a spectrophotometer by the amount of 490 nm absorbance. Formazan production is time dependent and proportional to the number of viable cells. CHO cells were cultured in 0.1 ml CHO media in 96-well flat-bottomed plates. Cultures were seeded at $1x10^4$ cells / well and allowed to attach overnight. After the indicated time of incubation with the appropriate medium, 20 μl reagent was added per well, and cells were incubated 1 hour before measuring absorbance at 490 nm. Background absorbance from the control wells was subtracted. Studies were performed in triplicate for each experimental condition (Soares R et al, 2007).

CELL CYCLE ASSAY

After treatments described above, cells (1.5×10^5/L cells) were suspended in medium, washed once with PBS, lyzed and stained with propidium iodide (PI) at room temperature for 15 min. DNA histograms were assayed by flow cytometry (Becton-Dickinson). In each sample, a minimum of 10 000 cells was counted and stored in list mode. Data analysis was performed using standard Cell Quest software (Becton-Dickinson).

WESTERN BLOT ANALYSIS

Western blot analyses of p27 were performed in Sh-treated cells and controls with a monoclonal antibody anti-p27 and normalized with Beta-actin expression (Santa Cruz Biotechnology, Santa Cruz, CA, USA), detected by chemiluminescent method and the resulting bands evaluated by imaging densitometer (BioRad GS-670). Experiments were repeated at least twice.

APOPTOSIS

TUNEL assay (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick endlabeling) was performed using the in situ cell death detection kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. Nuclei were counter-stained with DAPI (Roche Diagnostics, Basel, Switzerland). The percentage of TUNEL-stained nuclei was evaluated in relation to every DAPI-stained nuclei observed. Immunofluorescence was visualized under a fluorescence microscope (Olympus, BH-2, UK). The percentage of stained cells was evaluated by counting the cells stained with TUNEL divided by the total number of nuclei stained with DAPI at a magnification 200X field. One thousand nuclei were evaluated. Three independent experiments were performed. Apoptosis in cell

cultures was assessed with the *in situ* cell death detection kit, fluorescein (TUNEL technology) (Roche), analyzed by fluorescence microscopy (Soares R et al, 2007).

BCL-2 EXPRESSION ANALYSIS

The monoclonal antibody against Bcl-2 was used (Dako A/S, Glostrup, Denmark) conjugated with fluorescein isothiocyanate (FITC). Briefly, 2 x 10^5 cells were first permeabilized and fixed using a Fix and Perm kit (CALTAG, San Francisco, CA, USA), and then the cells were stained with Bcl-2/FITC. Data acquisition and analysis were performed by FACScalibur flow cytometer (Becton Dickinson) (Arden N et al, 2006).

CELL INVASION

Cell invasion was analyzed in 24-well Matrigel-coated invasion chambers (BD Biosciences, Bedford, MA) according to manufacturer's directions with the following modifications: hydrated chambers were transferred to a new 24-well plate containing medium with 20% serum. 5x10^5 cells (treated with Sh and control) were added to each chamber and incubated for 20 hours. Cells that did not pass through the filter were removed with a cotton swab prior to processing the filter with a Hema-3 staining kit (Fisher, Pasadena, CA) and mounting the filter on a microscope slide. Cells in five fields on the filter were photographed at 40x magnification and counted manually for each treatment (Soares R et al, 2007).

TUMOURIGENESIS

Nude mice were randomly divided into two experimental groups, group 1 with five animals and group 2 with three animals. CHO cells were suspended in 200 µl of PBS and

inoculated subcutaneous in the neck into the mice from group 1. Animals from group 2 were used as the control group; these animals were injected with CHO cells not treated with Sh.

After transplantation, the size of each tumour mass developed was measured with a calliper twice a week and tumour volumes were calculated as previously described (Tang et al., 2003). On day 22 after cell administration all animals were sacrificed under anaesthesia by intracardiac injection of pentobarbital (40 mg/Kg) and tumours were removed from each animal. The tumours were examined macroscopically, cut in sections with 4mm, fixed in 10% formalin and then embedded in paraffin. Haematoxylin and eosin (H&E) stained sections were prepared and examined to classify the neoplasic transformation.

INTRAVESICAL CARCINOGENESIS

Forty CD-1 mice were randomly divided into six experimental groups. Groups 1 and 3 (n=10) were Sh-instilled animals. Groups 2 (n=5) and 4 (n=5) were control animals with their bladders instilled with saline only. Groups 1 and 2 were sacrificed 20 weeks after treatment; group 3 and 4 were sacrificed 32 weeks after treatment. For the duration of the study, the mice's state of health was monitored daily.

For intravesical instillation of the parasite total antigen, 50 μg/ml of Sh was introduced directly into the urinary bladders of female mice via the urethra according to the procedure previously described [11]. Briefly, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). The bladder was catheterized via the urethra with a 24 gauge plastic intravenous cannula and the bladder mucosa was then traumatized to remove the umbrella cells of the urothelium by instilling 0.1 ml of 0.1N HCl solution for 15 seconds, neutralized with 0.1 ml of 0.1N KOH and flushed with sterile saline.

A total of 150 μl of Sh with a concentration of 50 μg/ml was instilled via the cannula. The dwell time after instillation was approximately 1 hour. The mice were turned 90 degrees every 15 minutes to facilitate whole bladder exposure to the Sh intravesically instilled. One hour after

the instillation, micturition of the mice was induced by light abdominal massage so that the duration of exposure was constant. Body temperature was maintained with a homoeothermic bandage. All the experiments described herein were carried out under aseptic conditions and performed once during this study.

All animals were sacrificed by intraperitoneal administration of sodium pentobarbital (130 mg/Kg). Complete necropsies were carefully conducted. All organs were examined macroscopically for any changes. The urinary bladders were inflated *in situ* by injection of 10% phosphate buffered formalin (150µl), ligated around the neck to maintain proper distension and then were immersed in the same solution for 12 hours. After fixation, the formalin was removed; the urinary bladder was cut into two strips, and routinely processed. Tissue sections (2 µm) from urinary bladder, spleen, liver and lung were stained with haematoxylin and eosin (HE) to characterize changes and inflammatory infiltrate.

MUTATION ANALYSIS OF *KRAS* GENE

Mutational analysis of *KRAS* codons 12, was performed by direct sequencing.

STATISTICAL ANALYSIS

For *in vitro* studies, data were expressed as mean ± SD. *t* test was used to assess the statistical significance of differences. $P < 0.05$ was considered statistically significant.

For animal experiments, a descriptive study was performed for all the variables included in the study. Statistical analysis was performed using the SPSS 12.0 statistical package for Windows (SPSS Inc. USA). The differences in incidence of lesions between groups were assessed by use of Fisher's exact probability test or the chi-square test. A $p<0.05$ was accepted as statistically significant.

B) EXPLOITATION OF THE HOST ENDOCRINE SYSTEM BY SCHISTOSOMES

HUMAN SAMPLES

Sera from 17 Schistosoma infected individuals and 8 controls were obtained from the CIBP (INSA-Porto). The individuals were living in endemic areas (Angola, Mozambique and Guinea). All sera were selected on the basis of positive urine and fezes egg counts. The controls were living in the same endemic areas.

HORMONE SERUM LEVELS

Estradiol, Testosterone, LH and SHBG were determined, using a solid phase competitive chemiluminescent enzyme immunoassay with the Immulite 2000 Analyser (DPC) according to the manufacturer's instructions. Briefly, the solid phase (bead) is coated with rabbit anti-hormone polyclonal antibody. The reagent contains alkaline phosphatase (bovine calf intestine) conjugated to the hormone analysed. The hormone-enzyme conjugate competes with the tested hormone in the patient sample for limited antibody-binding sites on the bead. The excess sample and reagent are removed by a centrifugal wash. Finally, chemiluminescent substrate is added to the bead and signal is generated in proportion to the bound enzyme.

CHEMICALS AND MATERIALS

CHO cells were cultured in CHO medium (Sigma Aldrich, Portugal) and MCF-7 and HCV29 cells were cultured in RPMI 1640 medium phenol red free (Sigma Aldrich, Portugal), with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, purchased in Invitrogen Life Technologies (Paisley, Scotland, UK). Estradiol (E2) was obtained from Sigma Aldrich

(Portugal) and the antiestrogen ICI 182,780 (ICI) was kindly provided by AstraZeneca (Portugal), both chemicals dissolved in 100% ethanol and added to cell culture medium.

PLASMIDS

Plasmids were used to transform DH5α *E. coli*. Individual colonies were isolated and plasmid miniprep was done using the Promega Miniprep Kit. The desired plasmid was amplified and isolated using the Qiagen maxiprep kit. The estrogen-responsive reporter plasmid, pERE-Luc, was kindly provided by Ming Tsai and Bert O'Malley (Baylor College of Medicine, Houston, TX) (Nawaz et al, 1999).

CELL CULTURE

CHO, MCF-7 and HCV29 cells were maintained at 37º C in a 5% CO2 humidified atmosphere. Cells were passaged every 5 days. Before treatments cells were serum-starved for 16 h. The cells were treated with Sh antigen for 24 h in serum-free conditions.

ESTRADIOL ASSAY

Estradiol levels from *in vitro* assays were determined using ELISA (Calbiotech, CA, USA) according to the manufacturer's instructions. Briefly, the E2 ELISA is based on the principle of competitive binding between E2 in the test specimen and E2-HRP conjugate for a constant amount of rabbit anti-Estradiol. In the incubation, goat anti-rabbit IgG-coated wells are incubated with E2 standards, controls, patient samples, Estradiol-HRP Conjugate Reagent and rabbit anti-Estradiol reagent at room temperature for 90 minutes. During the incubation, a fixed amount of HRP-labeled E2 competes with the endogenous E2 in the standard, sample, or quality control serum for a fixed number of binding sites of the specific E2 antibody. E2

peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of E2 in the specimen increases. Unbound E2 peroxidase conjugate is then removed and the wells washed. Next, a solution of TMB Reagent is added and incubated at room temperature for 20 minutes, resulting in the development of blue color. The color development is stopped with the addition of stop solution, and the absorbance is measured spectrophotometrically at 450 nm. A standard curve is obtained by plotting the concentration of the standard versus the absorbance.

LACTOFERRIN ASSAY

MCF-7 and HCV29 cells were cultured in 0.3 ml RPMI medium in 24-well flat-bottomed plates. Cultures were seeded at $5x10^4$ cells / well and allowed to attach overnight. Cells were then treated with 50 μg/ml of Sh for 24h. Lactoferrin in the supernatant of cells was determined using ELISA with an antibody specific for lactoferrin (Hycult, Netherlands) according to the manufacturer's instructions.

ISOLATION OF RNA

At defined time intervals MCF-7 and HCV29 cultured cells were harvested in Tripure reagent (Roche, Portugal). Total RNA was isolated from the cell material according to the manufacture instructions. RNA recovery and quality were checked by measuring the optical density ratio (260/280 nm) and its quantification was determined also by optical density (Nanodrop).

REAL-TIME PCR

Real-Time PCR was performed in MCF-7 and HCV29 cultures. For this purpose cDNA was obtained using the Promega cDNA kit. For each sample, 1.0 µg of RNA was reverse transcribed in a reaction volume of 20 µl in the presence of 10 mM deoxynucleotide triphosphate and 2 µl RT-PCR enzyme mix. Taqman GeneExpression assays (Applied biosystems) were used for gene-specific primers and probes for ER alpha (ESR1, PE Applied Biosystems, USA) and ER beta (ESR2, PE Applied Biosystems, USA). Both target gene transcripts were normalized to the endogenous control GAPDH mRNA (PE Applied Biosystems, USA) content and to the control sample. Quantifications were done in triplicate in 7500 Real-Time PCR System (PE Applied Biosystems, USA).

TRANSIENT TRANSFECTION

CHO cells were plated for transfection in 24-well plate and incubated for 24 h at 37º C with 5% CO2 the day before transfection. The cells were transfected with 0,75 μg ERE-Luc plasmid per well and 0,3 μg pCMV-β (which constitutively produces β-galactosidase) for normalization. Cells were transfected by using Lipofectamine (Invitrogen, Carlsbad, California, USA) according to the manufacturer's recommended guidelines. Cells were washed and fed with CHO medium and subsequently treated with 50 μg/ml of Sh, 10^{-9} E2 and 10^{-9} M ICI. Concentrations for E2 and ICI used were based on previous studies (Soares R et al, 2003). As a control, cells were treated with vehicle alone (ethanol). After 24 hr, cells were harvested and cell extract were prepared for luciferase assay. Luciferase activity was determined using an enhanced luciferase assay kit (BD Biosciences, USA) in a scintillation counter (Amersham Biosciences, USA). Cell extracts were assayed for β-galactosidase activity as described previously (MacGregor and Caskey, 1989), and the luciferase units were normalized to β-galactosidase activity from the cotransfected pCMVβ internal reference plasmid.

MASS SPECTROMETRY ANALYSIS

LC-MS analysis were carried out using a Thermo Finnigan Surveyor LCQ DECA XP MAX quadrupole ion trap mass spectrometer, utilizing electrospray ionization, coupled to a LC of Thermo Finnigan. UV detection was achieved at 280 nm and the column was a Merck Purospher STAR RP-18e 125 cmx4.6 (3 μm), equipped with a Merck Lichrocart pre-column (Merck, Germany).

The mobile phases consisted of water/acetonitrile/acetic acid mixtures, 95:5:o.5 for A and 45:55:0.5 for B. The following gradient was 0-5 min, 100% A; 5-10 min, linear gradient from 100% to 80% A; 10-15 min, 80% A; 15-50 min, linear gradient from 80% to 40% A; 50-65 min, 40% A; 65-75 min, linear gradient from 40% A to 100% B. The flow rate was 0.5 mL/min and for MS2 experiments, the collision energy was optimized at 30%.

ESTROGEN RECEPTOR IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed in 5 μm sections attached to silane coated slides that were dried for 48 to 72 hours at 37 ºC before use. After dewaxing in xylene and rehydration through graded alcohol to water, antigens were retrieved using microwaving at 850 W in 10nM citrate buffer, pH 6,0, for 10 minutes and allowed to cool. After a brief wash in distilled water endogenous peroxidise activity was quenched by incubation with 3% hydrogen peroxide for 10 minutes. Nonspecific binding sites were blocked with 10% normal rabbit serum (Dako, Glostrup, Denmark) diluted in phosphate buffered saline (PBS). Sections were incubated with primary mouse monoclonal antibody against ER (Zymed, San Francisco, CA, U.S.A.) in a humidified container for 18 hours at 4ºC. After washing with the appropriate PBS for 20 minutes the sections were incubated with biotinylated rabbit anti-mouse Igs (Dako; 1:400) for 30 minutes at room temperature. After another wash with buffer for 20 minutes specimens were incubated with Vectastain ABC Elite avidin-biotin complex linked horseradish peroxidise reagent for 30 minutes at room temperature in a humidified chamber. After a further wash with buffer for 30

minutes immunoreactivity was visualized with exposure to 3,3'-diaminobenzidine substrate (Vector Laboratories, Peterborough, United Kingdom) for 5 minutes. Sections were briefly counterstained with Mayer's hamatoxylin (Sigma).

IMMUNOHISTOCHEMISTRY ANALYSIS

The distribution of positively stained cells in the urothelium, urethral epithelium and smooth muscle cells was determined as a percentage of positive cell nuclei counted. The intensity of the reaction was recorded as follows: negative (-), weak (1+), moderate (2+), and strong (3+).

STATISTICAL ANALYSIS

For hormone serum levels statistical tests were performed using the WassarStats: web site for Statistical Computation software. The values of Estradiol for various groups were compared using Mann-Whitney non-parametric test for the significance of the difference between the distributions of two independent samples.

For *in vitro* experiments data were expressed as mean ± SD. *t* test was used to assess the statistical significance of differences. $P < 0.05$ was considered statistically significant.

For animal experiments statistical analysis was performed using Microsoft Excel Analysis ToolPak for Windows. The differences in expression of Estrogen Receptor between groups were assessed by use of the chi-square test. A $p<0.05$ was accepted as statistically significant.

RESULTS AND DISCUSSION

The results are documented and discussed in detail in the section Papers I to IX. In this section, a summary will be presented highlighting the most interesting findings according to the specific aims.

A) MECHANISMS UNDERLYING THE ASSOCIATION BETWEEN S. HAEMATOBIUM AND SQUAMOUS CELL CARCINOMA (SCC) OF THE BLADDER

I - *SCHISTOSOMA HAEMATOBIUM* IMMATURE WORMS INDUCE GRANULOMATOUS-LIKE IMMUNE REACTION AND HEPATIC FIBROSIS SIMILAR TO PARASITE EGGS (PAPER I)

We observed hepatic lesions induced by *S. haematobium* immature male worms in the golden hamster. We characterized these lesions and the nature of the local immune response by examining the hepatic inflammatory infiltrate. We demonstrated the induction of hepatic fibrosis and hepatitis, characteristic of egg granulomas, induced by male adult worms of *S. haematobium* alone. Periworm granuloma-like formation occurred in infected animals with immature worms lodged in portal veins of the liver (Figure 4B).

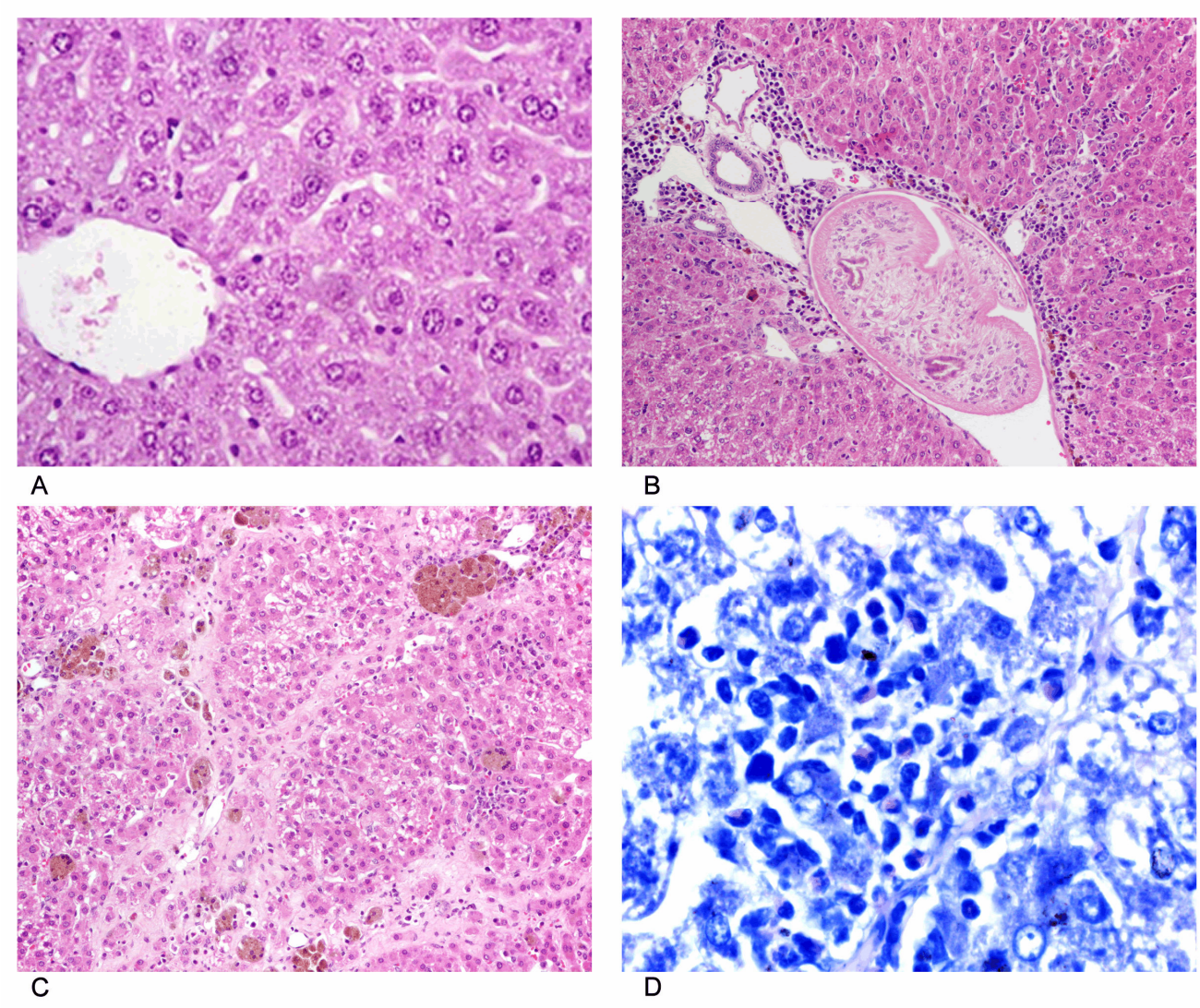


Figure 4: Histopathology of parasitic liver. A) Control; B) Liver section of *Schistosoma haematobium*-infected hamster, H&E, 400X; C) Macrophages with abundant bilharzial pigment, H&E, 200X; D) Eosinophiles infiltrate, Wright, 1000X

Granulomas of schistosome-infected hamsters are composed of numerous macrophages, eosinophilic granulocytes, lymphocytes and fibroblasts. Similar dominant cell types were characterizing the granulomatous-like reaction around the worms, thus closely resembling the hepatic granulomas induced by schistosome eggs although without the concentric layers (Figures 4B, 4C and 4D). Macrophages, fibroblasts and lymphocytes, and eosinophils were found to infiltrate along the outer layer of the hepatic induced granuloma-like.

These histopathology appearances represent an early advanced feature of granuloma formation.

Histopathology of liver sections infected hamsters revealed the presence of *S. haematobium* immature worms and fibrous perihepatitis. In addition, we recorded infiltration of eosinophils, plasma cells, macrophages with bilharzial pigment and fibroblasts. These features are the characterization of granulomas. Because these features, fibrosis and multinuclear cell formation are both hallmarks of granuloma development (Hogan et al, 2002) our experimental single sex infection model revealed that immature worms had striking effects of granuloma-like development characterized by cellularity and/or cell composition (monocytes/macrophages, neutrophils and eosinophils) and fibrosis (Figure 5).

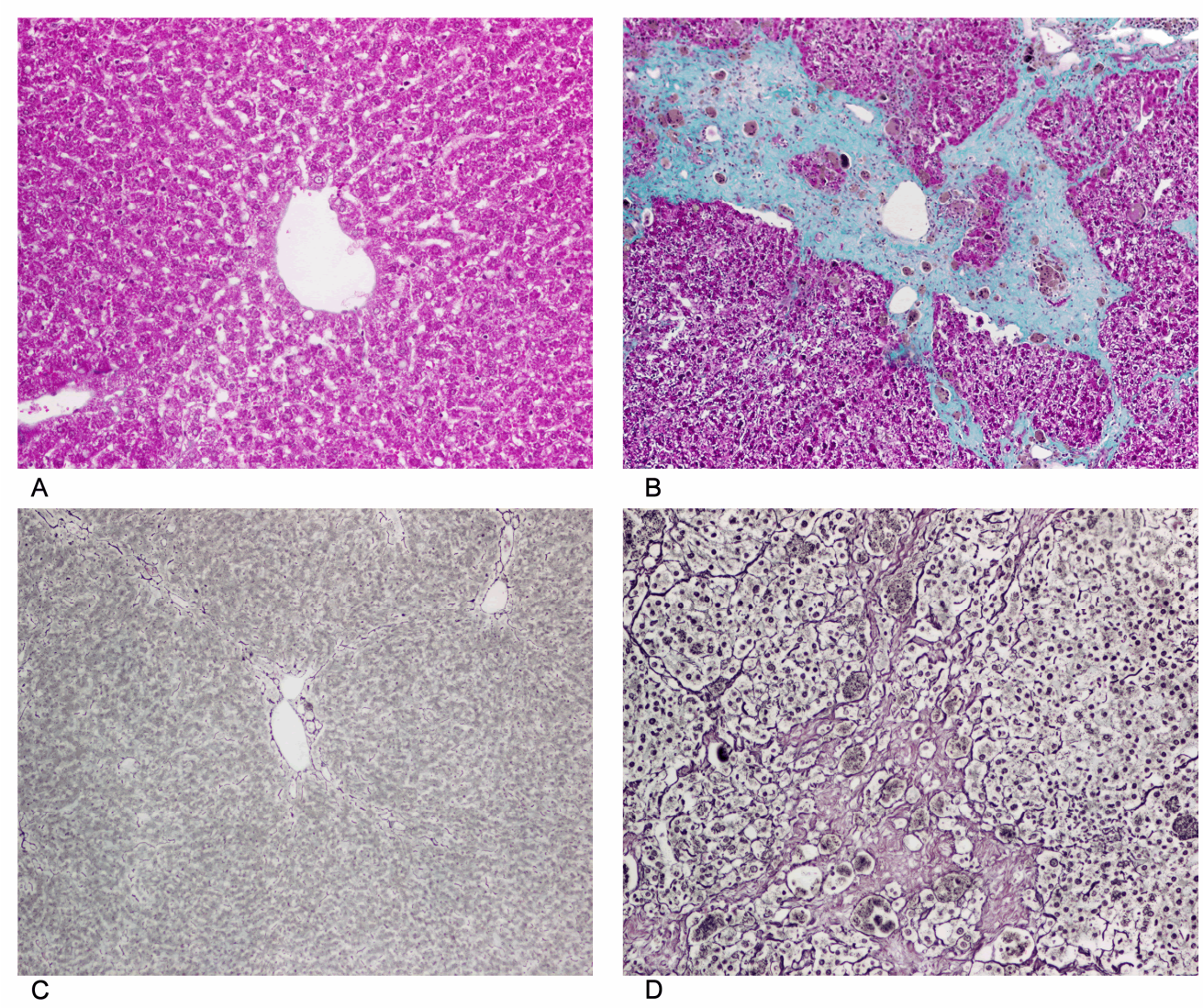


Figure 5: Liver fibrosis A) Hamster liver section with normal Masson Trichrome stain, Trichrome stain, 200X; B) Portal-portal fibrous bridging and fibrous septa, connecting portal areas to each others and lobule centers, Masson's trichrome stain was used. Collagen fibres show blue staining, 400X. C) Normal reticulin pattern, reticulin stain, 100X; D) Black lines between hepatocytes, hamster with hepatic fibrosis, reticulin stain, 200X;

There is no report in the literature that associates *S. haematobium* immature worms to hepatic fibrosis. But in schistosomiasis *schistosoma* Baki et al (1998) reported hepatic fibrosis and histopathological lesions in mice experimentally infected with male Schistosoma *schistosoma*. These authors revealed that, from the 25th week post-infection, a diffuse fibrosis

affected the main branches of the portal vascular system following the host inflammatory reaction, associated with the proliferation of myofibroblasts in situ (Baki et al, 1998). An increase of fibrotic deposit occurred during chronic unisexual infection suggesting that antigenic substances secreted by adult schistosomes, in the absence of any eggs, might initiate periportal and perisinusoidal fibrous reaction.

II - *S. HAEMATOBIUM* TOTAL ANTIGEN INCREASES THE PROLIFERATION, DECREASES THE APOPTOSIS AND INDUCES INVASION OF EPITHELIAL CELLS *in vitro* (PAPER II, III AND VIII)

Cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis (Hanahan and Weinberg, 2000). To begin investigating the effect of Sh (*Schistosoma haematobium* total antigen) on cell viability and proliferation, CHO cells were seeded on 96 well plates, starved overnight, treated with increasing concentrations of Sh for 24 h, cultivated for 24, 48 and 72 hours and then analyzed by MTS assay (Fig. 6). The growth curve shows that treated cells proliferated significantly faster and more than control cells.

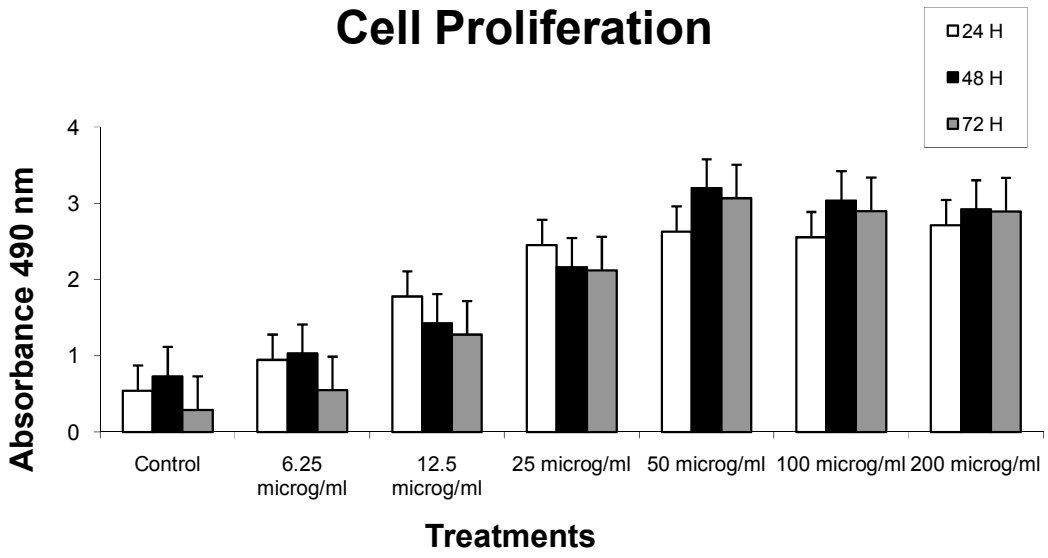


Figure 6: Cell proliferation assay of *S. haematobium* total antigen treated cells. The experiments were done in triplicate. The growth curve shows that treated cells proliferated significantly faster and more than control cells. ($p<0.01$; Control vs. 50 μg/ml).

We hypothesized that increased cell proliferation is the result of cell cycle progression driven by Sh, and therefore analysed cell cycle progression with flow cytometry (Fig. 7). This method revealed that the cell growth induction by Sh was a result of an increase of G2/M- and S-phase and a decrease of G0/G1-phase (Fig. 7B), as compared to control (Fig. 7A).

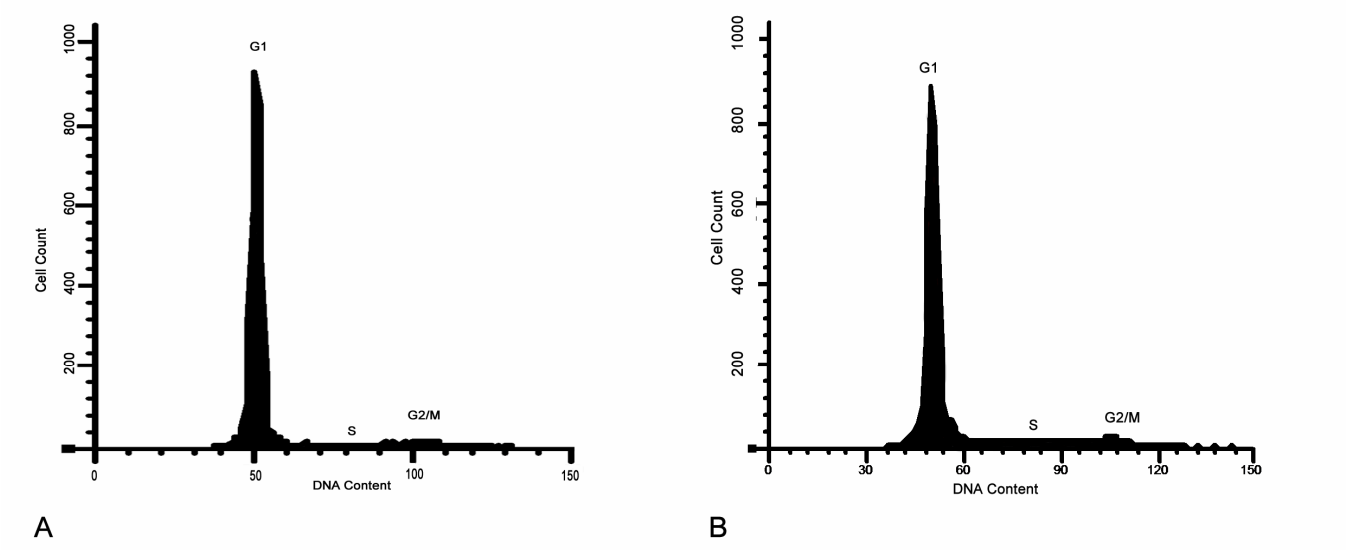


Figure 7: Effects of treatment with Sh on cell cycle distribution 48 h after treatment. (A) Control cells. (B) Flow cytometry revealed that the cell growth induction by Sh was a result of an increase of G2/M- and S-phase and a decrease of G0/G1-phase, as compared to control.

We further examined whether Sh affect the expression of cell cycle-related proteins. Expression of p27, a cyclin dependent kinase inhibitor (CDKI) was analyzed in CHO cells after Sh treatment. Immunoblotting for p27 was performed in CHO cells. p27 protein was down-regulated in Sh-treated CHO cells (Fig. 7C).

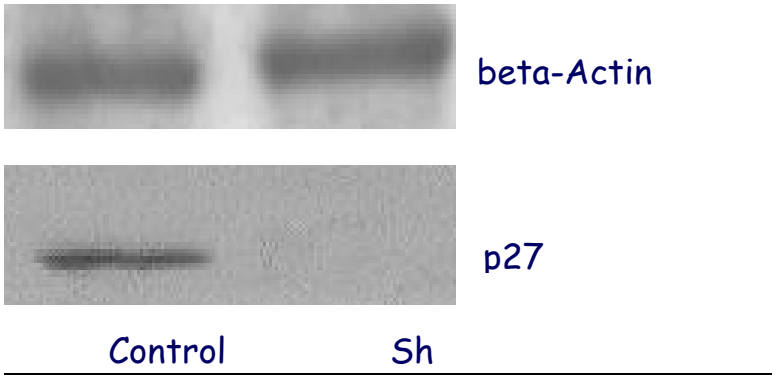


Figure 7C: Expression of p27, a cyclin dependent kinase inhibitor (CDKI) was analyzed in CHO cells after Sh treatment. Immunoblotting for p27 was performed in CHO cells. p27 protein was down-regulated in Sh-treated CHO cells.

In accordance, the results of Thuwajit et al (2004) reported that excretion/secretion (ES) products from *Opistorchis viverrini*, a liver fluke associated with cholangiocarcinoma, induced cellular proliferation in mouse fibroblasts NIH-3T3 cells. Kim Y et al (2008) used the human epithelial cell line HEK293 to examine the *in vitro* effect of ES products of *Clonorchis sinensis*, another liver fluke also associated with cholangiocarcinoma, on the growth of bile duct epithelial cells. The cells proliferated in the presence of ES products in a dose-dependent manner (Kim Y et al, 2008).

To analyze apoptosis, CHO cells were seeded on 96 well plates, starved overnight, treated with increasing concentrations of Sh for 24 h, cultivated for 24, 48 and 72 hours and then analyzed by TUNEL assay (Fig. 8). CHO cells showed an increasing number of apoptotic cells per field in the control (Fig. 8A) compared to cells treated with 50 μg/ml of Sh (Fig. 8B). Cell counts show that treated cells markedly decreased apoptosis in comparison to control cells.

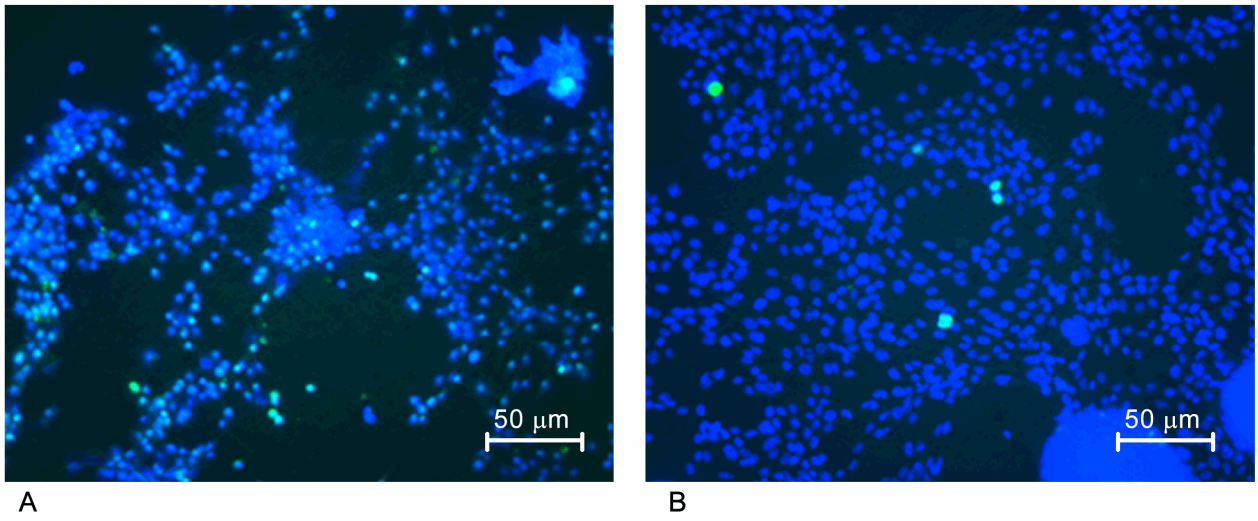


Figure 8: Apoptosis of Sh-treated cells analysed by TUNEL. The experiments were done in triplicate (A) Microphotographs of apoptotic cells of controls and (B) Sh-treated cells. Both panels have the same magnification. CHO cells, showing an increasing number of apoptotic cells per field in the control compared to cells treated with 50 μg/ml of Sh. The white bars represent 50 μm.

Since the Bcl-2 family of proteins is regarded as key regulator of apoptosis, the influence of Sh on Bcl-2 expression levels in CHO cells was subsequently investigated. Bcl-2 is perceived as anti-apoptotic (Miyake et al., 2001). In this study Sh exposure led to significant increase in Bcl-2 expression levels when compared to controls (Fig. 9). The percentage of cells Bcl-2 positive in the control group (Fig. 9a) was 50 % whereas in the Sh-treated group was 53 % (Fig. 9b).

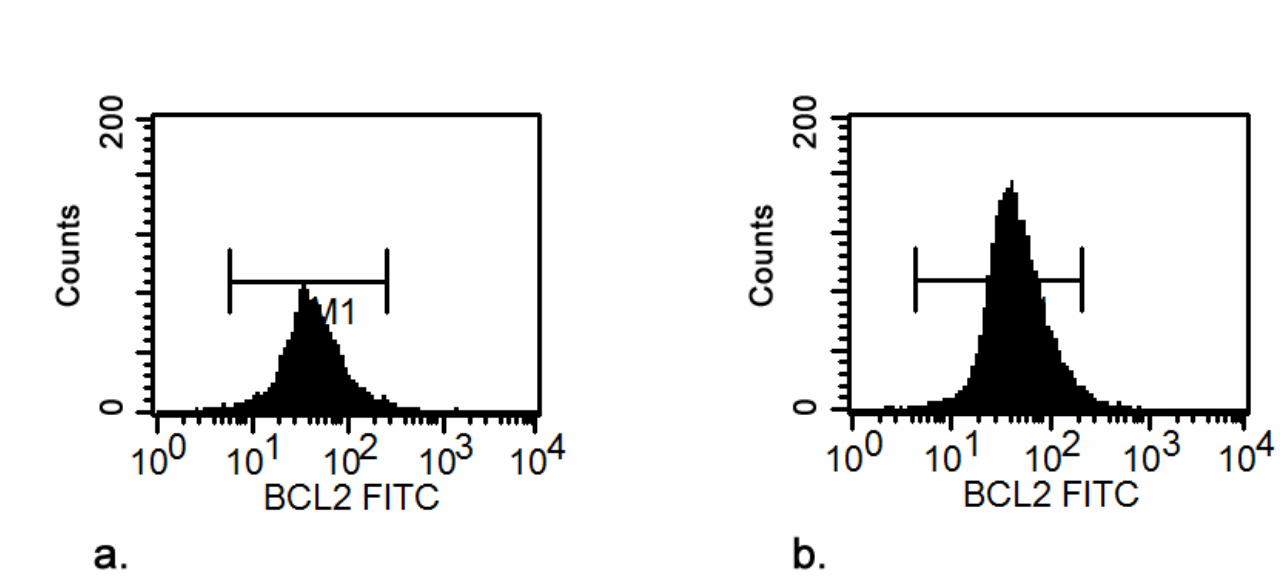


Figure 9: Histogram of CHO cells expressing BCL-2 by flow cytometry. Control (left panel) and Sh-treated cells (right panel). In this study Sh exposure led to significant increase over controls in Bcl-2 expression levels.

In agreement, apoptosis is rarely observed in either *O. viverrini*-infected hamster liver or in biliary cells that are co-cultured with flukes *in vitro*. Biliary cell damage by *O. viverrini* likely stems from the actions of oxygen radicals such as nitric oxide (NO). NO not only induces DNA damage but has been reported to mediate DNA repair inhibition. Moreover, NO has also been demonstrated to inhibit apoptosis. All of these manifestations facilitate carcinogenesis. Anti-apoptosis has been described in infection-associated cancers such has those caused by *Helicobacter pylori*. In the case of *O. viverini* infection, DNA damage is caused in biliary epithelial cells while apoptotic mechanisms are dysregulated, resulting in genetic alterations which may become fixed, leading to malignant transformation (Sripa B et al, 2007).

Cell motility and tissue invasion was suggested to be one of the essential alterations in cell physiology that collectively dictate malignant growth (Hanahan and Weinberg, 2000). To test whether Sh induces invasion *in vitro*, we measured invasion using Boyden chambers (Fig. 10). Membranes in the chambers are coated with Matrigel to mimic basement membrane. Movement of cells through the membrane requires not only migration but also degradation of

matrix, a critical step in the invasion process. Our results show that treatment of cells with Sh stimulated invasion (Fig. 10B) compared to controls (Fig. 10A).

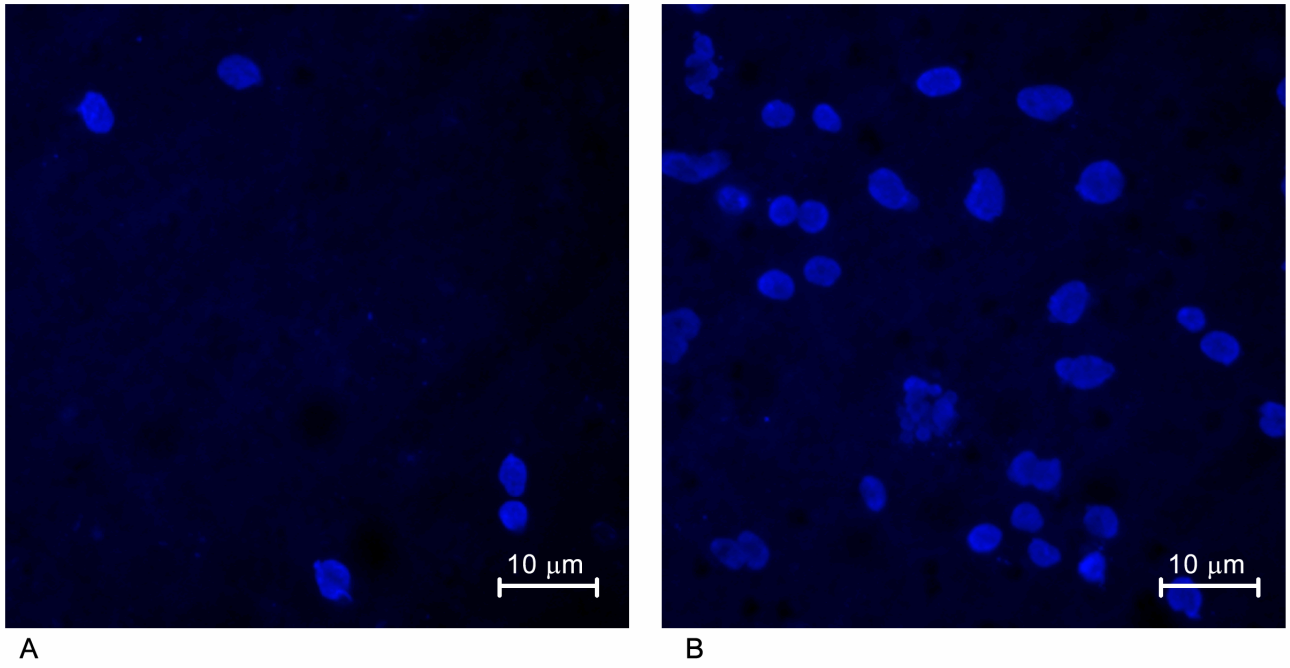


Figure 10: Effect of Sh on cell invasion **(A)** Microphotographs of invasive cells of controls and **(B)** Sh-treated cells. Both panels have the same magnification. Note the increased number of cells stained by Dapi in the Sh-treated cells. The white bars represent 10 μm.

Understanding the mechanisms that lead to the invasion of host tissues by a growing population of neoplastic cells is a major task of cancer research (Santra et al., 1997). In the present work we have used *in vitro* assays to evaluate migration and invasion of Sh-treated cells and found them increased in comparison to controls. As Oliveira and collaborators (2006) have stated, the Matrigel invasion assay used in this study is by no means the equivalent of invasion in the *in vitro* situation. These investigators have repeatedly emphasized that all elements of the ecosystem need to be taken into account when analyzing the phenotype of cells. Nevertheless, the Matrigel assay we used contains some of the elements of the *in vitro* ecosystem of invasion, such as collagen type IV, laminin, and entactin. Furthermore, this assay

also covers some of the cellular activities implicated in invasion, like three-dimensional migration through matrices and proteolysis (Oliveira MJ et al, 2006). We have demonstrated that Sh induce invasion of CHO cells through the use of Matrigel invasion assay. This could lead to extracellular matrix degradation and subsequent invasion of cancer cells, suggesting a role for Sh in later stages of bladder carcinogenesis.

Taken together, the effects observed in Sh-treated cells seem to be interconnected. Through its effects in cell biology, *S. haematobium* total antigen is likely to participate in a number of carcinogenesis mediated processes, such as increased cell proliferation and loss of p27, decreased apoptosis and increased expression of Bcl-2, and increased migration and invasion, all of which are processes needed for cancer cell survival. The effects of Sh on the cell cycle may contribute to the high proliferation rate and accumulation of genetic changes during schistosomiasis associated tumorigenesis. Loss of p27 may be the reason for the uncontrolled proliferation seen in Sh-treated cells and could be involved in bladder cancer-associated schistosomiasis.

III - *S. HAEMATOBIUM* TOTAL ANTIGEN INDUCES TUMORIGENESIS (PAPER III AND VIII)

We have used a xenograft model to show that *Schistosoma haematobium* total antigen (Sh) has the potential to induce tumour development. All mice injected with Sh- treated CHO cells developed a solid, large multilobulated mass in interscapullary region. These tumours grew very fast and ulcerated overlying the skin (Figure 11).

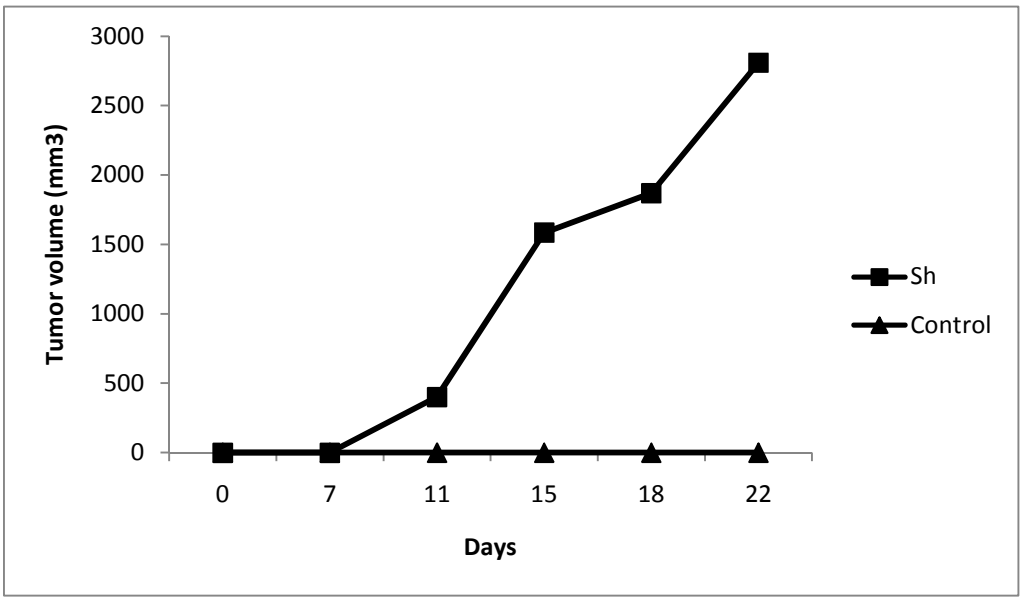


Figure 11: Analysis of the tumour volume induced by control Chinese hamster ovary (CHO) and *Schistosoma haematobium* total antigen (Sh) treated CHO in nude mice. Tumour volumes were measured at the indicated days after injection. Each point represents the mean of five individual determinations.

No macroscopic changes were observed in the interscapular region in the control group. The cut surface of the tumours was white and firm. Microscopic observation of these tumours showed a pleomorphic and fusocellular neoplasia with intensive inflammatory infiltrate, predominantly lymphocytes and neutrophils. We have also identified round cells with prominent nucleus, and exuberant nucleolus, with a histiocytic pattern and frequent mitosis. Necrotic foci were seen in these tumours. All tumours corresponded to sarcomas. There were no tumour metastases.

The morphology of CHO cells is both epithelial-like and fibroblast-like. Our results suggest that Sh may induce the transformation of the fibroblast-like component of CHO cells into sarcomas. This result is not surprising since it has been shown that egg granulomas isolated from livers of mice infected with Schistosoma *schistosoma* or S. *schistosoma* and cultured *in vitro* released a fibroblast-stimulating substance which stimulated proliferation in resting dermal fibroblasts (Wyler and Tracy, 1982). This liver pathology is the terminal event of a

complex pathophysiologic cascade involving interactions between fibroblasts and both host and parasite products (Lammie et al., 1986) and is associated with prominent accumulations of fibroblasts (Dunn et al., 1986), reinforcing the present results.

IV - *SCHISTOSOMA HAEMATOBIUM* TOTAL ANTIGEN INDUCES DYSPLASIA IN CD-1 MICE NORMAL UROTHELIUM (PAPER IV, V AND VIII)

We have used CD-1 mice to show that *Schistosoma haematobium* total antigen has a carcinogenic ability. We investigated the effects of *S. haematobium* total antigen in CD-1 mice normal bladders after intravesical instillation of the parasite antigens. The bladders were analyzed histopathologically 20 and 40 weeks after treatment. Intravesical instillation of *S. haematobium* total antigen induces the development of urothelial dysplasia and inflammation. The bladder histopathological findings observed in all groups are given in Table 1 and Figure 12.

Table 1: Histopathological findings in the Sh instilled bladders and controls.

Group	n	W	N	U	I	L	M	D
Group 1 (Sh treated)	10	20	7/10 (70%)	5/10 (50%)	9/10 (90%)	9/10 (90%)	0/10 (0%)	3/10 (30%)
Group 2 (Control)	10	20	10/10 (100%)	9/10 (90%)	2/10 (20%)	2/10 (20%)	0/10 (0%)	0/10 (0%)
Group 3 (Sh treated)	10	40	0/10 (0%)	0/10 (0%)	10/10 (100%)	9/10 (90%)	4/10 (40%)	7/10[a] (70%)
Group 4 (Control)	10	40	10/10 (100%)	10/10 (100%)	3/10 (30%)	3/10 (30%)	0/10 (0%)	0/10 (0%)

n, number of animals; W, weeks between treatment and sacrifice; N, normal urothelium; U, presence of umbrella cells; I, inflammatory infiltrate; L, infiltration of lymphocyte; M, infiltration of mastocytes; D, dysplasia.

[a] (p=0.001, Group 3 Vs. Group 4)

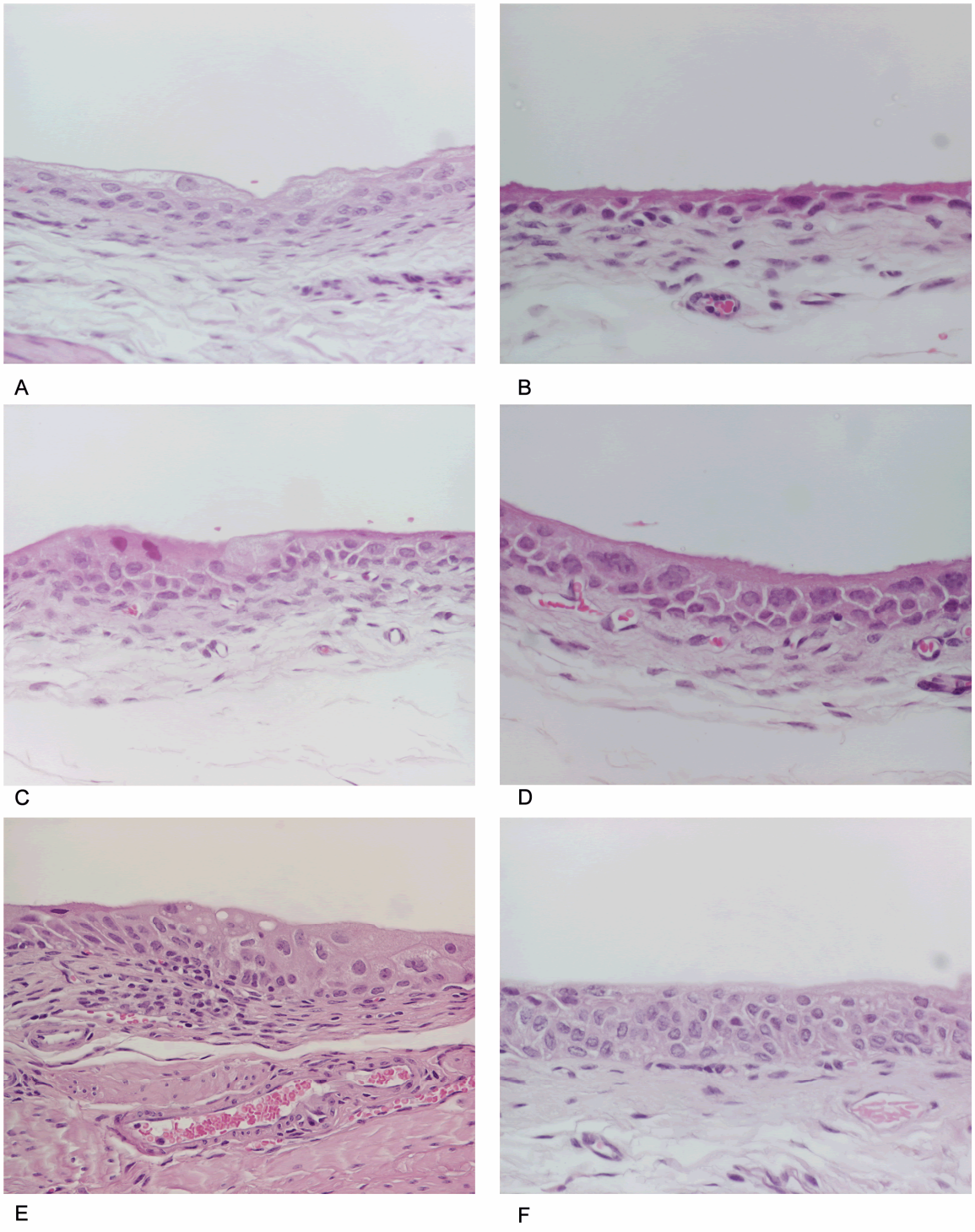


Figure 12: Histopathological findings in animals after 40 weeks of treatment. A – Control (400X); B – Dysplasia (600X) and C – Dysplasia (400X); D - Urothelium displaying a lesion with characteristics of reactive atypia (600X); E - Inflammatory infiltrate and dysplasia (400X); F - Urothelium displaying a lesion with characteristics of simple hyperplasia with reactive atypia (600X).

Bladder histopathology of animals instilled with Sh was characterized by extensive diffuse or nodular infiltrate composed predominantly of lymphocytes admixed with variable number of eosinophils, and by absence of umbrella cells, nodular hyperplasia and dysplasia. The nodular hyperplasia exhibiting budlike proliferated urothelial cells extending into the lamina propria. After 20 weeks of treatment 30% of these animals displayed urothelial dysplasia a LGIUN (Low Grade Intra Urothelial Neoplasia), whereas after 40 weeks of treatment we found 70% of the bladders with this LGIUN, a non-invasive malignant flat lesion in the urothelium of these animals. No bladder histopathological changes were observed in animals from control groups. No microscopic changes were seen in the spleen, lung, kidney and liver in samples obtained from all animals.

Our results suggest that Sh induced the malignization of the urothelium. Although helminths have been implicated in the etiology of human cancer, the knowledge of the mechanisms by which parasites induce malignant transformation of the host cells is unclear. *Schistosoma haematobium* is considered to be carcinogenic to humans, with sufficient evidence for its role in causing carcinoma of the bladder (Parkin et al, 1994; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1994). According to Herrera and Ostrosky-Wegman (2001), parasites might initiate carcinogenesis by direct action of genotoxic factors, either secreted by them or produced during the inflammatory response. Activation of inflammatory cells may cooperate to induce cancer in many cases of infection- and inflammation-associated cancers (Ohshima et al, 2003a; Ohshima et al, 2003b).

V - *SCHISTOSOMA HAEMATOBIUM* TOTAL ANTIGEN INDUCES *KRAS* GENE MUTATIONS IN THE BLADDERS OF CD-1 MICE (PAPER V AND VIII)

We showed above that intravesically instillation of Sh gave rise to a high incidence of urothelial dysplasia, a non-invasive malignant flat lesion, in 70% of the tested subjects after 40 weeks of treatment. We were able to investigate this effect further and show that 20% of the

dysplastic bladders presented a mutation in codon 12 of *KRAS* gene. Two (out of 10) of the bladders with dysplasia presented a *KRAS* mutation in codon 12 of exon 2 (Fig. 13). We concluded from these results that the parasite extract of *S. haematobium* has carcinogenic ability possibly through oncogenic mutation of *KRAS* gene.

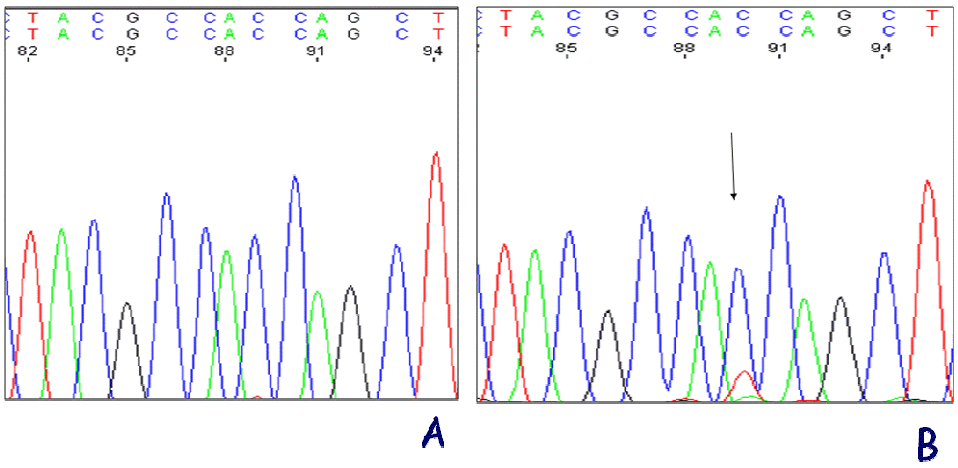


Figure 13: *KRAS* exon 2 sequencing results. (A) control, (B) dysplastic bladder with a mutation at codon 12 (resulting in a Gly12Asp mutation).

We cannot state at this point that *KRAS* mutation that we detected in dysplastic bladders induced by Sh treatment is an initiating event. The presence of this mutation could have one of two explanations: either the parasite total antigen has carcinogenic molecule(s) sufficiently genotoxic and, consistent with the mutation observed, caused direct attack on the ras gene leading to uncontrolled proliferation of the cells and causing dysplasia in the bladders treated with the parasite antigen; or the parasite antigens are only mitogenic for the urothelial cells causing increased proliferation that resulted in the *KRAS* activation via non-carcinogen-mediated pathways. Few studies were carried out to investigate the role and frequency of activated ras in schistosomiasis-associated bladder cancer (Badawi, 1996). The mutations were not detected in two previous studies (Fugita et al, 1987; Ramchurren N et al, 1995). These findings indicate that the advanced stage of schistosomiasis-associated bladder cancer may not

involve a high frequency of detectable mutations within the ras genes. It is known that more than 90% of the SCC in the schistosome-infected bladder was advanced (stages T3 and T4) at the time of diagnosis. However, this does not exclude the possibility that activation in ras could be an important factor in the initiation as well as promotion and progression of schistosomiasis-associated bladder cancer (Badawi, 1996).

B) EXPLOITATION OF THE HOST ENDOCRINE SYSTEM BY SCHISTOSOMES

I – *SHISTOSOMA HAEMATOBIUM* PRODUCES AN ESTRADIOL-RELATED MOLECULE (PAPER VI)

In a series of patients infected with *S. haematobium*, we attempted to identify and quantify the sex hormones estradiol (E2), testosterone and luteinizing stimulating hormone (LH) in their sera by ECLIA (electrochemoluminescence) (Immulite 2000 Analyser, DPC; Terrytown, New York) (Table 2).

Table 2. Sera of *Schistosoma*-infected patients and controls were subject to analysis by ECLIA for the sex steroids estradiol (E2), testosterone, luteinizing hormone (LH). Typical reference values (range) are shown for sex and age.

Group	Sex	Age (yr)	E2 (pg/ml)	Range	Testosterone (ng/dl)	Range	LH (mUI/ml)	Range
1 (Sh)	Female	4	**62.8**	0-22	<15.0	2-10	0.114	<2.5
1 (Sh)	Male	12	**30.8**	0-25	77.5	5-500	1.79	0.2-8.0
1 (Sh)	Male	14	**79.8**	0-25	363	5-500	1.89	0.2-8.0
1 (Sh)	Male	17	**45.7**	0-25	724	>200	5.89	1.4-7.7
1 (Sh)	Male	17	**31.9**	0-25	535	>200	7.65	1.4-7.7
1 (Sh)	Male	20	**68.3**	<56.0	982	262-1593	2.87	1.4-7.7
1 (Sh)	Male	22	**65,1**	<56.0	1225,2	262-1593	3,61	1.4-7.7
1 (Sh)	Male	21	**67,6**	<56.0	266,1	262-1593	4,9	1.4-7.7
2 (Sm)	Male	30	**76,1**	<56.0	1027	262-1593	2,48	1.4-7.7
2 (Sm)	Male	28	**104,3**	<56.0	923,9	262-1593	5,58	1.4-7.7
2 (Sm)	Male	25	**77,1**	<56.0	1084,3	262-1593	3,00	1.4-7.7
2 (Sm)	Male	32	**75,8**	<56.0	1499	262-1593	1,60	1.4-7.7
2 (Sm)	Male	20	**152,6**	<56.0	1488,1	262-1593	1,90	1.4-7.7
2 (Sm)	Male	35	**84,9**	<56.0	683,4	262-1593	N.D.	1.4-7.7
2 (Sm)	Male	24	**88,21**	<56.0	740,4	262-1593	4,12	1.4-7.7
2 (Sm)	Male	25	**58,1**	<56.0	698,7	262-1593	3,84	1.4-7.7
2 (Sm)	Male	23	**59,2**	<56.0	1038,2	262-1593	1,86	1.4-7.7
3 (Ctrl)	Male	29	44,7	<56.0	422,8	262-1593	2,72	1.4-7.7
3 (Ctrl)	Male	24	39,3	<56.0	385,1	262-1593	7,07	1.4-7.7
3 (Ctrl)	Male	26	48,8	<56.0	642	262-1593	1,79	1.4-7.7
3 (Ctrl)	Male	28	49,3	<56.0	548,6	262-1593	1,19	1.4-7.7
3 (Ctrl)	Male	28	38,6	<56.0	464,5	262-1593	1,50	1.4-7.7
3 (Ctrl)	Male	22	35,9	<56.0	379,3	262-1593	3,16	1.4-7.7
3 (Ctrl)	Male	24	50,1	<56.0	702,1	262-1593	N.D.	1.4-7.7
3 (Ctrl)	Male	27	43,5	<56.0	433,7	262-1593	1,7	1.4-7.7

Sh: *S. haematobium*-infected patients, Sm: *S. mansoni*-infected patients, Ctrl: Controls, N.D.: Not determined

The individuals were living in an endemic area and all sera were selected on the basis of positive egg counts. We observed that in all of these patients, serum levels of estradiol were significantly elevated in comparison with non-infected controls living in the same endemic areas. One of the patients, a 4-yr-old girl, had E2 levels similar to those at puberty. The other hormones presented normal levels. The fact that LH was normal in these patients is surprising, and contradicts the normal feedback effect of the endocrine system, in which LH should lower the excess E2 (March et al, 1979)

We hypothesized that the elevated levels of estradiol observed in Schistosoma-infected patients were produced by the parasite itself and, therefore, we analyzed the total antigen extract of the parasites. We observed the expression of an estradiol-related molecule. The estradiol production analyzed by ELISA in the *S. haematobium* total antigen was 15 pg/ml of antigen extract. We found that S. *schistosoma* also produces this estradiol-related molecule, although in a smaller concentration (13 pg/ml). Our results clearly show the production of a related molecule of estradiol by Schistosoma. Several authors have attempted and succeeded in identifying hormones in this group of parasites. For example, Nirde et al. (1983) demonstrated the presence of the insect molting hormones, ecdysone and 20 hydroxyecdysone, in S. *schistosoma* by the combined use of radioimmunoassay and high pressure liquid chromatography (HPLC). These authors thus established for the first time that S. *schistosoma* synthesizes steroid hormones. Later, the same team of researchers (Nirde et al., 1984) demonstrated that these molecules could be used for parasite diagnosis. The significance of our findings is supported by the knowledge that schistosomes have estradiol receptors (Barrabes et al, 1986). In mammals, estradiol regulates diverse physiological processes in reproductive tissues and in mammary, cardiovascular, bone, liver, and brain tissues. The effects of estradiol are mediated via its receptors ER, which are members of the nuclear receptor superfamily of ligand-activated transcription factors that control these physiological processes, in large part through the regulation of gene transcription. A molecule able to bind estradiol has been previously described in schistosomes. It could be used by the parasite, as a likely

mechanism for the protective effect of this hormone in infected mice and hamsters (Barrabes et al, 1986).

Recent experimental evidence suggests that schistosomes can not only evade immune responses actively, but also exploit the hormonal microenvironment within the host to favor their establishment, growth, and reproduction (Escobedo et al., 2005). It is generally believed that infection by schistosomes leads to modifications in the hormonal status of their hosts and that this is the result of a secondary reaction of the host to infection, one that is not directly mediated by parasite-derived factors (Mendonça et al., 2000).

II - *S. HAEMATOBIUM* PRODUCES ESTROGENIC MOLECULES THAT ARE ABLE TO DOWN-REGULATE ER ALPHA AND ER BETA AND REPRESS ER TRANSCRIPTIONAL ACTIVITY (PAPER VII AND IX)

Expression of ER alpha and ER beta were analyzed in MCF-7 cells after estradiol, ICI and Sh treatment. Real-Time PCR analysis for ER alpha, ER beta, and endogenous control gene GADPH were carried out by using RNA derived from either vehicle-treated (control), estradiol-treated (E2), ICI-treated (ICI) or Sh-treated in MCF-7 cell cultures. Incubation with estrogen clearly increased both ERalpha and ER beta expression (Figure 14). Furthermore, both ER isoforms were downregulated by Sh treatment in this cell culture, either alone or in the presence of ICI in comparison to control (Figure 14).

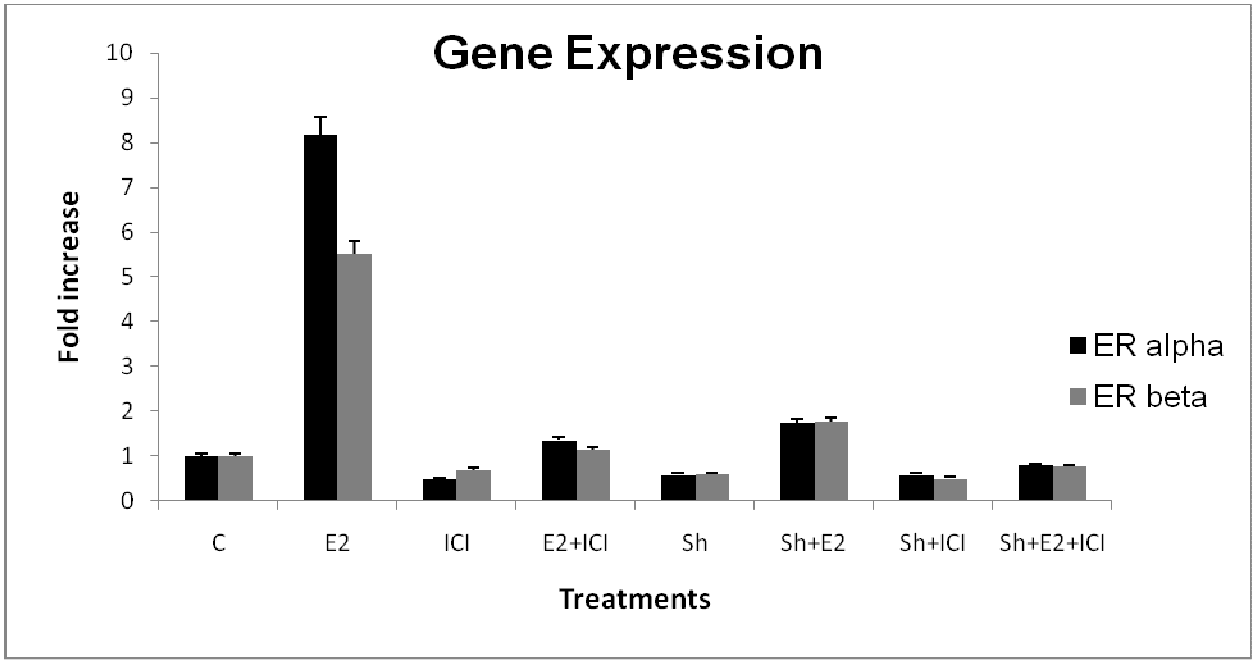


Figure 14: Real-Time PCR for ER alpha and ER beta in MCF-7 cells. Bars correspond to fold increase of ER alpha and ER beta gene expression after normalization to endogenous control gene GAPDH and control sample. Results are representative of three independent experiments. Statistically significant differences in ER alpha and ER beta gene expression were found between Sh-treated and vehicle-treated MCF-7 cells ($p < 0.05$ Sh vs. control).

Nuclear hormone receptors regulate gene expression by interacting directly with DNA response elements. To confirm Sh-mediated down-regulation of ER we carried out transfection of CHO cells with a reporter gene construct containing an estrogen response element (ERE). As shown in Figure 15, Sh effectively reduced the transcriptional activity of the ER. When cells are treated simultaneously with Sh and ICI there is a synergistic effect in the reduction of luciferase activity. Sh was also able to significantly reduce ER transcriptional activity in the presence of E2 (Figure 15) as revealed by comparing Sh+E2 with E2 results.

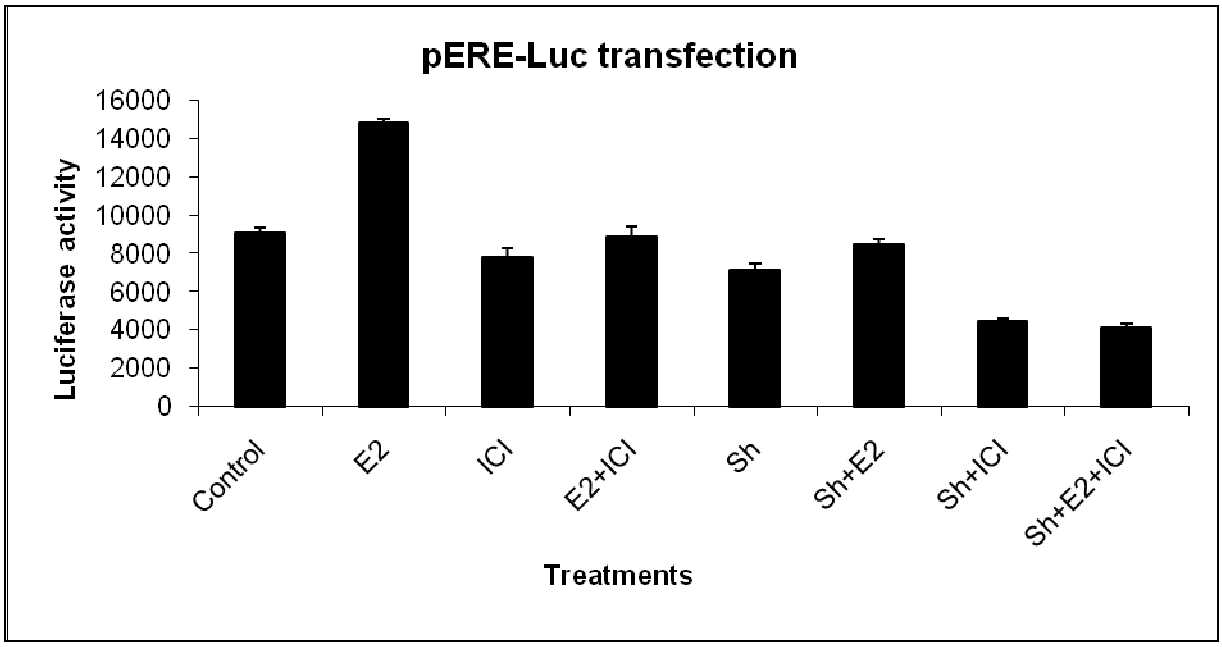


Figure 15: Role of Sh on Activation of Estrogen Responsive Element (ERE) was assessed by Luciferase Assay. CHO cells were transfected with a pGL3 reporter construct containing an ERE linked to luciferase, and incubated with 10^{-9} M E2, 10^{-9} M ICI or 50 μg/ml Sh afterwards. Reporter gene transactivation was evaluated 2 d after transfection. Luciferase activity induction was measured. Incubation with Sh or in the presence of ICI resulted in a statistically significant decrease in luciferase activity compared to control ($p < 0.05$ Sh vs. control; Sh+ICI vs. Control). Co-incubation of Sh together with E2 further reduced E2 effects on ER transcription activity. Bars represent mean values of three distinct assays. Error bars represent SD. Experiments were performed in triplicate.

Liquid chromatography coupled to electrospray ionization mass spectrometry (LC-ESI-MS) was used to identify new estrogenic molecules produced by *Schistosoma haematobium*. Figure 16 depicts chromatograms obtained for biological samples by LC-ESI-MS. As seen, both the *schistosoma* extract (Figure 16A) and the plasma from a shistosoma-infected individual (Figure 16B) present four main peaks with retention times of approximately 20, 22, 24 and 26 minutes. Interestingly, these four peaks were not observed for the plasma from a healthy donor (Figure 16C).

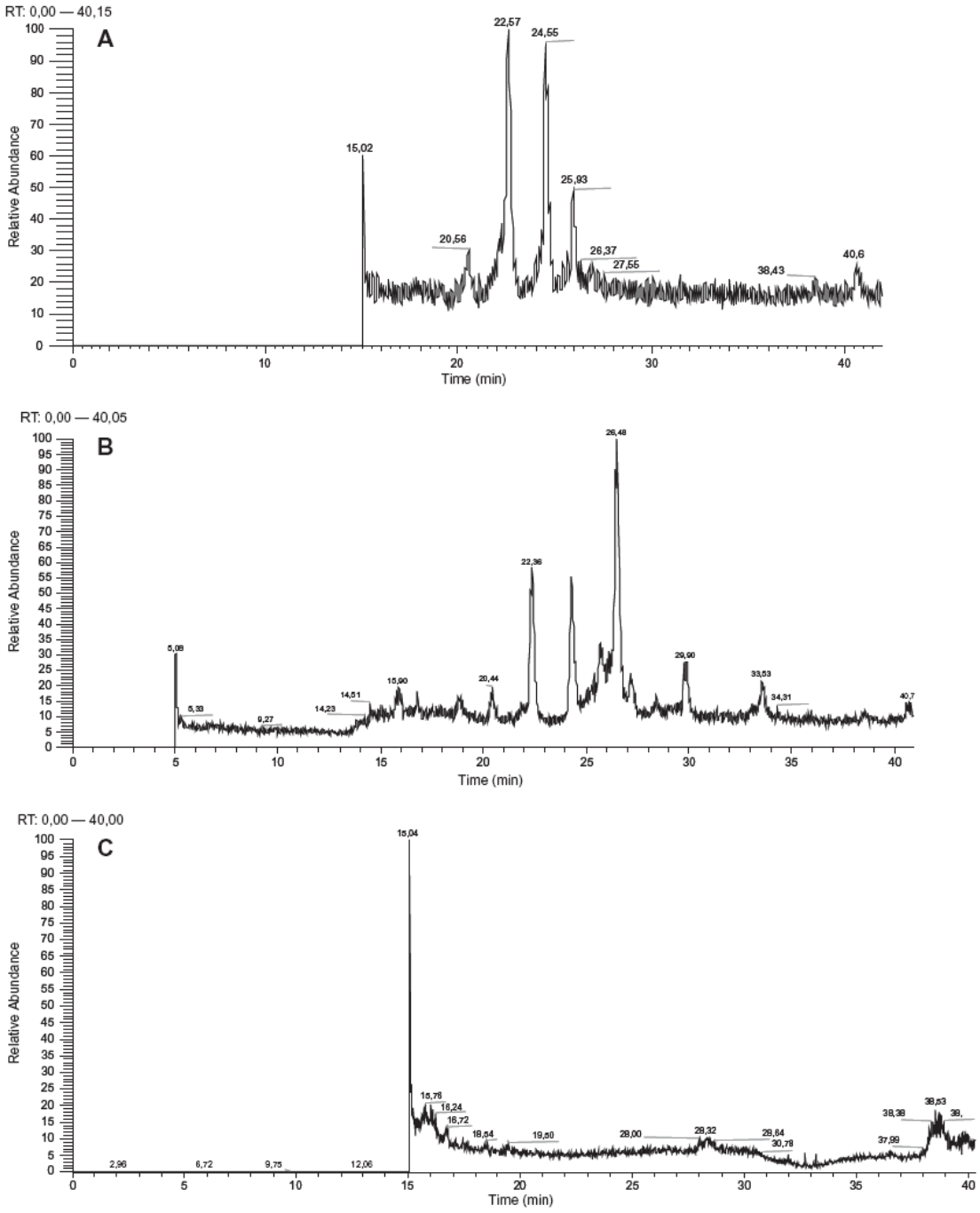
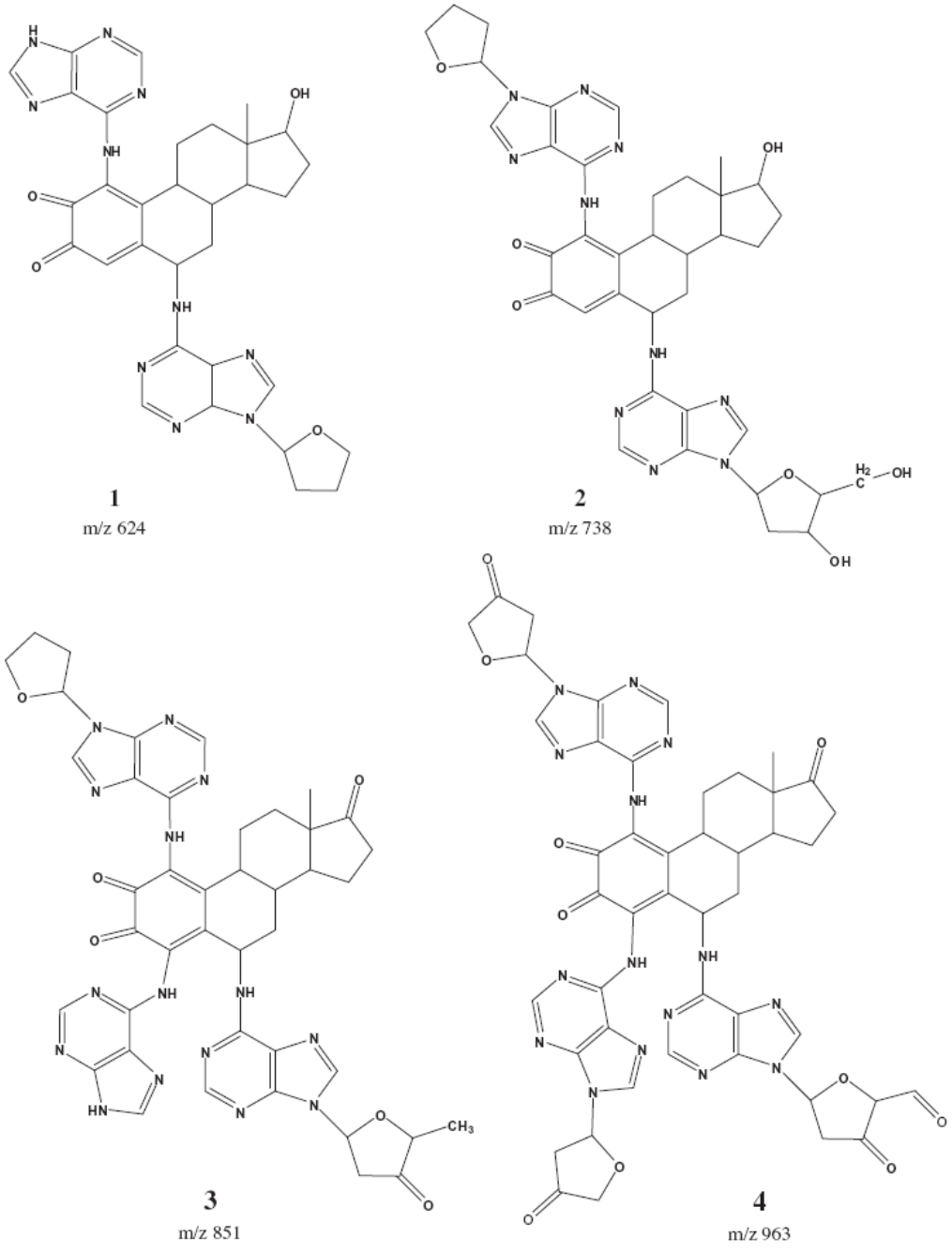


Figure 16: LC-ESI-MS analysis of *schistosoma* extract (A), serum from a *schistosoma*-infected individual (B), and serum from a healthy donor (C).

The structural assignment for the four components is depicted in Scheme 1. The species detected after MS2 fragmentation of components 1-4, gave further support to the molecular structures of the components.



Scheme 1: Components 1, 2, 3 and 4 detected by LC-ESI-MS.

These studies document that Sh represses the activation of ER-induced estrogen responsive element. Sh expresses estrogenic molecules that interact with ER by downregulating and repressing ER transcriptional activity. These experiments show that there is a direct interaction of Sh with ER and that this interaction is ligand-dependent. The ability of Sh to decrease transcriptional activity by the ER was observed with a reporter gene construct containing an ERE. The data presented suggests that Sh modulates the concentration of ER transcripts in the estrogen responsive MCF-7 cells, by promoting its down-regulation in a similar way as the estrogen inhibitor ICI. Sh clearly downregulates ER alpha and ER beta in MCF7 cells. The estrogen receptor (ER) exists in two isoforms ER alpha and ER beta with a different distribution in the body and different functions, which are not clearly identified yet (Zimmerman and Angerer 2007). Physiological effects of estrogen are mediated through both ER alpha and beta. Importantly, ER alpha- and ER beta-dependent pathways regulate distinct and largely nonoverlapping sets of genes. Whereas ER alpha is essential for most of the estrogen-mediated increase in gene expression, ER beta mediates the large majority (nearly 90%) of estrogen-mediated decreases in gene expression (O'Lone et al, 2007). Additionally, it is possible that Sh-induced ER alpha and beta down-regulation serves to control physiological responses in estrogen target tissues, which ultimately serves to limit the expression of estrogen responsive genes by the parasite.

These results are consistent with the existence of an estrogenic molecule that antagonizes the activity of estradiol. This molecule was identified and characterized by Mass Spectrometry. New estrogenic molecules previously unknown are present in schistosome worm extracts and sera of schistosome infected individuals. The detection method developed by LC-MS analysis specific for estrogenic molecules, revealed that these new estrogens are present in schistosome worm extracts. At the light of these findings, we believe that the species detected on the LC-MS analysis of both the *schistosoma* extract and the plasma of a *schistosoma*-infected human were formed by reactions of estrogen-quinones with DNA. An interpretation of these findings was built at the light of well-established metabolic pathways for estrogens. It is known from the literature that estradiol is mainly metabolised by (i) oxidation of the hydroxyl function at the C17 position (via steroid dehydrogenase) to yield estrone, and (ii) hydroxylation

via cytochrome P450 enzymes, occurring preferentially on C2, C4 and C16 (Bolton JL et al, 1998 and Rathahao et al, 2000). When hydroxylation takes place on the steroid aromatic ring A, catechol-estrogens are produced (Iverson SL et al, 1996). Furthermore, the proposed molecular structure of these new estrogenic molecules explains the cellular effects of Sh demonstrated in this paper. These molecules show a putative binding affinity with ER and they probably are able to antagonize estradiol activity and downregulate ER.

Finally, to our knowledge there are no studies in the literature reporting the presence of genes involved in estrogen pathways in *S haematobium*. As the genome sequences of the closely related African schistosome *S. mansoni* (Berriman M et al, 2009) and the more distantly related Asian species *S. japonicum* (The Schistosoma *schistosoma* genome sequencing and functional analysis consortium, 2009) are now available we can expect that schistosomes will express all the genes necessary to synthesize estrogenic molecules. In this regard, it is noteworthy that the genomic information suggests the presence of an integral hypothalamic-pituitary-thyroid in *S. japonicum*. It was also confirmed that *S. japonicum* has receptors for steroid hormones such as progestin, progesterone and estrogen. In addition it possesses intricate pathways for processing steroid hormones to form other sex hormones. For example, there are putative enzymes present that could convert the female hormones progesterone and pregnenolone to estriol, oestrone, androsterone and testosterone. Hence, schistosomes might use these pathways during their parasitic existence (The Schistosoma *schistosoma* genome sequencing and functional analysis consortium, 2009).

III - *SCHISTOSOMA HAEMATOBIUM* TOTAL ANTIGEN DOWN-REGULATES ER ALPHA AND ER BETA IN HCV29 NORMAL UROTHELIAL CELLS AND DOWN-REGULATES ER EXPRESSION IN THE BLADDERS OF CD1 MICE (PAPER IX)

Expression of ER alpha and ER beta were analyzed in HCV29 cells after estradiol, ICI and Sh treatment. Real-Time PCR analysis for ER alpha, ER beta, and endogenous control gene GADPH were carried out by using RNA derived from either vehicle-treated (control), estradiol-treated (E2), ICI-treated (ICI) or Sh-treated in HCV29 cell cultures. Incubation with

estrogen clearly increased both ER alpha and ER beta expression (Figure 17). Furthermore, both ER isoforms were downregulated by Sh treatment in this cell culture, either alone or in the presence of ICI in comparison to control (Figure 17). Interestingly, Sh incubation extensively reduced ER alpha and ER beta expression, even in the presence of estrogen ($p < 0,05$ Sh+E2 vs. E2).

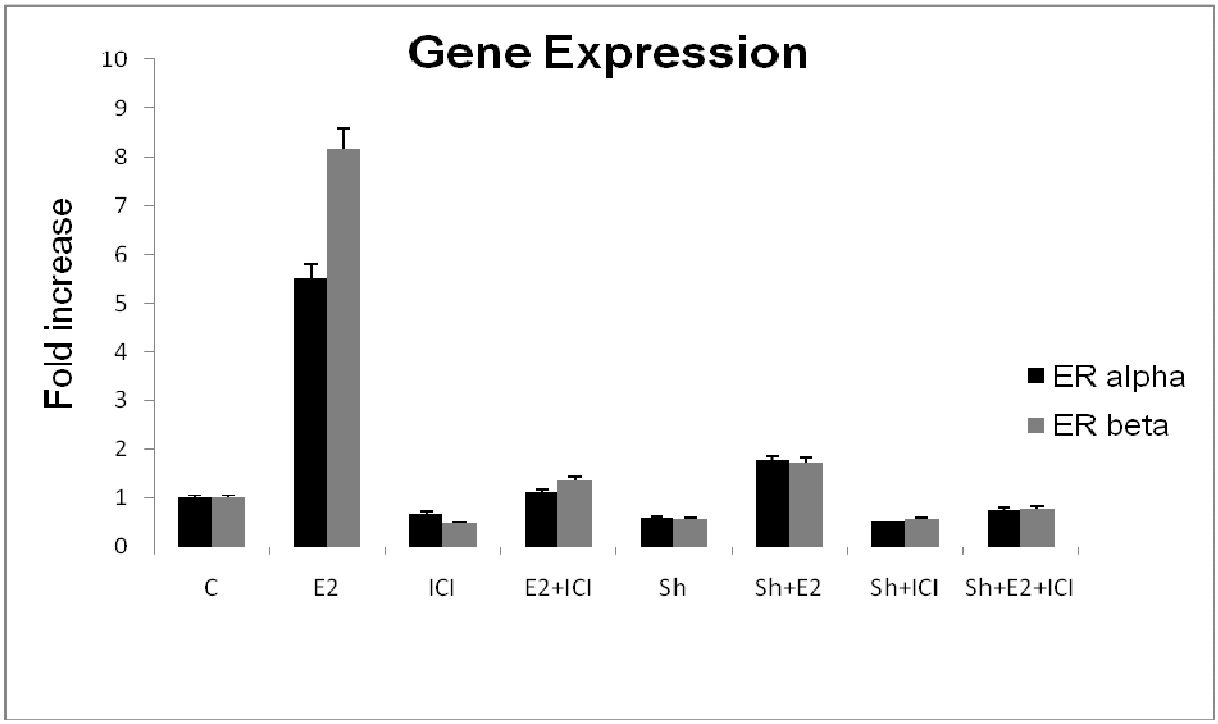


Figure 17: Real-Time PCR for ER alpha and ER beta in HCV29 cells. Bars correspond to fold increase of ER alpha and ER beta gene expression after normalization to endogenous control gene GAPDH and control sample. Results are representative of three independent experiments. Statistically significant differences in ER alpha and ER beta gene expression were found between Sh-treated and vehicle-treated HCV29 cells ($p < 0.05$ Sh vs. control).

A total of 30 bladders, 10 controls and 20 treated animals, were analysed for the ER expression. The urothelium was always negative for ER protein expression in all animals of the 4 groups. The controls of Group 2, sacrificed after 20 weeks, showed positive staining in the urethral epithelium and in the smooth muscle cells (SMC) in 80% (4 out of 5) of the bladders

(Table 3, Figures 18A, 18B, 18C and 18D). The treated bladders were negative for ER expression in the urethral epithelium (Table 3, Figure 18E) and SMC (Table 3, Figure 18F) in 60% (6 out of 10) of the bladders of mice belonging to Group 1 which were sacrificed after 20 weeks. The bladders of the animals in this group that were considered positive for ER expression (40%) exhibited a week immunostaining. The treated animals of Group 3, sacrificed after 40 weeks, were ER negative for urethral epithelium and SMC in 50% (5/10) of the bladders. The other half showed a pattern of staining similar to the positive bladders of Group 1 (Table 3). Group 4, controls sacrificed after 40 weeks, showed positive ER expression in the urethral epithelium and in the smooth muscle cells (SMC) in 60% (3 out of 5) of the bladders.

Table 3: Expression of ER in Sh-instilled bladders and controls.

	Evaluated structures	Number of samples showing the stated estrogen-receptor immunoreactivity (%)			
Group		Negative	Weak (+)	Moderate (++)	Strong (+++)
1 (n=10)	Urothelium	10(100)		-	-
	Urethra	6(60)	4(40)	-	-[a]
	Smooth muscle	6(60)	4(40)	-	-[b]
2 (n=5)	Urothelium	5(100)	-	-	-
	Urethra	1(20)	-	3(60)	1(20)
	Smooth muscle	1(20)	-	3(60)	1(20)
3 (n=10)	Urothelium	10(100)	-	-	-
	Urethra	5(50)	5(50)	-	-[c]
	Smooth muscle	5(50)	5(50)	-	-[d]
4 (n=5)	Urothelium	5(100)	-	-	-
	Urethra	2(40)	-	2(40)	1(20)
	Smooth muscle	2(40)	-	2(40)	1(20)

[a] (p=0,01 urethra Group 1 vs. Group 2); [b] (p=0,01 smooth muscle Group 1 vs. Group 2); [c] (p=0,03 urethra Group 3 vs. Group 4); [d] (p=0,03 smooth muscle Group 3 vs. Group 4)

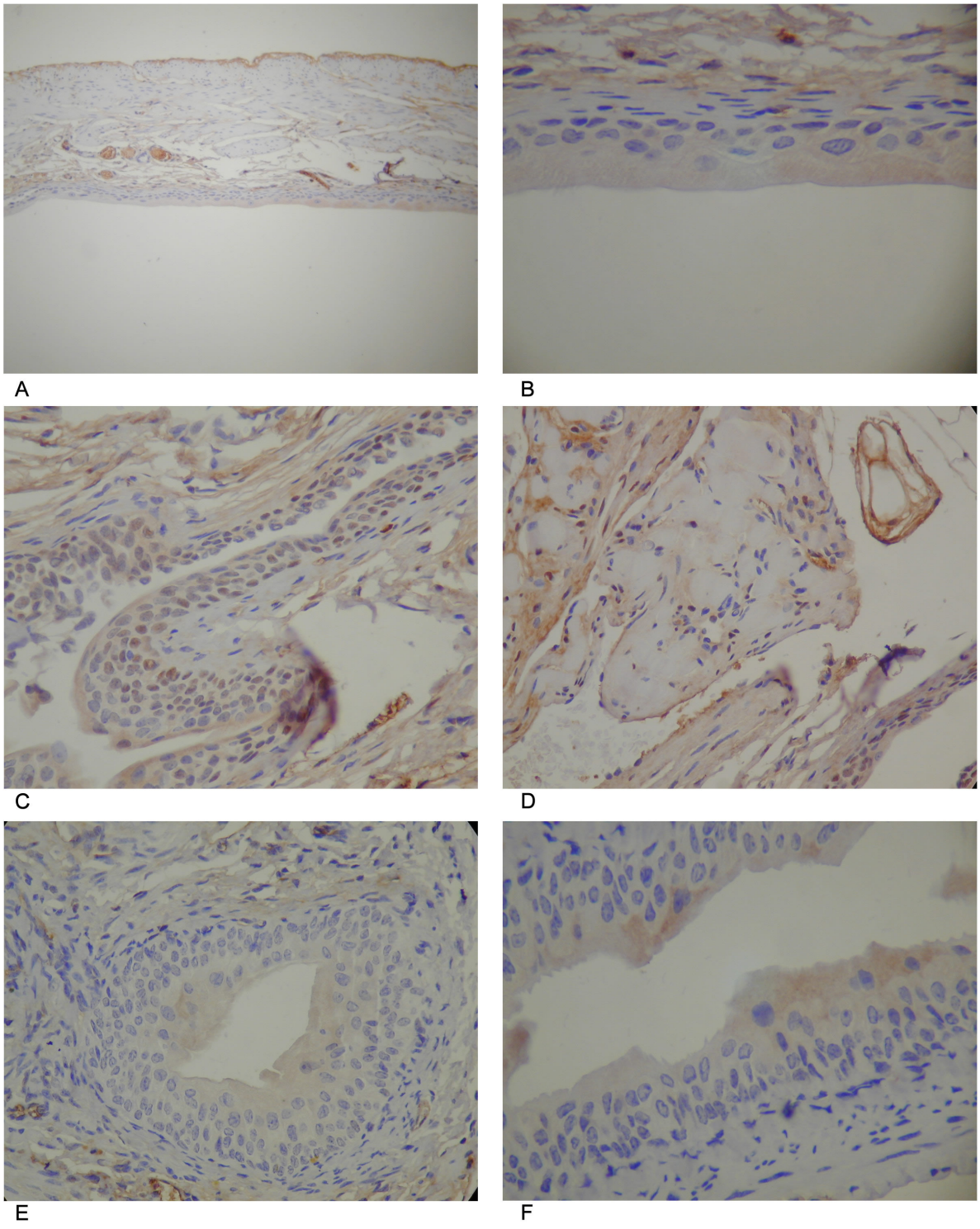


Figure 18: Immunohistochemistry staining for ER in the bladders of mice. A and B, urothelium of a control animal showing negative staining for ER; C, urethral epithelium of a control animal showing stained nuclei (400x); D, SMC of a control animal also with positive staining (400x); E, urethral epithelium of Sh-treated mouse with negative expression (400X); F, SMC lining the urethral epithelium of Sh-treated mouse also negative for ER expression (400x).

This work addresses the regulation of expression of *S. haematobium* antiestrogenic activity as an important mechanism used by the parasite to influence ER transcription. The benefit that this ER repression may provide to the parasite could be explained by the following hypothesis: estradiol treatment as been shown to increase the smooth muscle density of the bladder in female rats (Lin Y et al, 2006). In our study we observed downregulation of expression of ER in the smooth muscle of Sh-treated bladders. During infection with *S. haematobium* the parasite eggs must cross the bladder mucosa to reach the lumen of the bladder to be excreted, in order to survive and continue its life cycle. A thinner smooth muscle layer in the bladder mucosa of infected individuals could possibly increase the number of eggs released.

On another level, the repression of ER during *S. haematobium* infection could be a collateral damage of the infection. Nevertheless ER repression could have implications in the long term sequella of bladder cancer associated with this infection. As we described above, four estrogenic molecules have been identified in the parasite and in the sera from infected patients that are capable, according to their molecular structure, to bind ER and exert the repression effect observed. May the prolonged exposure to estrogens correlate with an increased risk for bladder cancer? At the light of these findings, we propose that the molecules detected on the LC-ESI-MS analysis of both the Schistosoma extract and the plasma of Schistosoma-infected patients could be carcinogenic. The molecules detected on the LC–MS analysis of both the *schistosoma* extract and the plasma of a *schistosoma*-infected human were formed by reactions of estrogen-quinones with DNA. The quinone forms generated by metabolic activation of catechol estrogens are highly electrophilic species. Some authors reported that these species are able to covalently bind DNA bases, forming stable adducts (Stack DE et al, 1996; Cavalieri EL et al, 1997; Akanni A et al, 1997a; Akanni et al, 1997b). These adducts can be considered as estrogen-bound metabolites generated *in vitro* that are produced by nucleophilic attack of the DNA base on different positions of the steroid ring (Cavalieri EL et al, 1997; Akanni A et al, 1997a; Akanni et al, 1997b; Farmer PB et al, 1995). Catechol estrogen-3,4-quinone adducts of DNA have been proposed as endogenous initiators of breast, prostate and other human cancers (Zahid M et al, 2006). In such cases, oxidation of the catechol estrogen metabolites 4-

and 3-hydroxyestradiol would lead to formation of, respectively, estradiol-3,4-quinone and estradiol-2,3-quinone, whose reactions with DNA have shown to be much more reactive and correlated with the carcinogenicity and mutagenicity of their precursors (Zahid M et al, 2006). The apparently higher reactivity of estradiol-3,4-quinone with DNA may be possibly due to its longer half-life, providing extended reaction time with DNA (Iverson SL et al, 1996).

The carcinogenic effect of this estrogen adduct-mediated pathway of the estrogenic molecules present in *S. haematobium* extracts could explain the link between this parasite and squamous cell carcinoma of the bladder, as well as our findings described above regarding proliferation, apoptosis, tumorigenesis, dysplasia and gene mutations caused by extracts of *S. haematobium* in normal cells and normal urothelium.

CONCLUSIONS

The main conclusions of the studies included in the present thesis are the following:

A) MECHANISMS UNDERLYING THE ASSOCIATION BETWEEN S. HAEMATOBIUM AND SQUAMOUS CELL CARCINOMA (SCC) OF THE BLADDER

In these studies we used *S. haematobium* total antigen on epithelial cells and explored its functions using *in vitro* and *in vivo* experimental models:

1. *S. haematobium* total antigen induced increased proliferation, decreased apoptosis, and induced migration and invasion of normal epithelial cells in culture. In addition, the parasite extract has also the potential to induce tumour development, assessed by the use of a nude mice xenograft model.

2. According to our findings, *S. haematobium* total antigen in CD-1 mice normal bladders after intravesical instillation of the parasite antigen, induced alterations in the urothelium of these animals consistent with dysplasia and inflammation. In these animals, we also found that the parasite extract of *S. haematobium* has carcinogenic ability possibly through oncogenic mutation of *KRAS* gene.

B) EXPLOITATION OF THE HOST ENDOCRINE SYSTEM BY SCHISTOSOMES

1. *S. haematobium* total antigen expresses estradiol-related molecules that down regulate Estrogen Receptor alpha and beta in estrogen responsive cells. These estrogens are also present in the sera of *Schistosoma*-infected individuals, and they have the ability to repress Estrogen Receptor transcriptional activity.

2. The estrogenic molecules present in *S. haematobium* extracts could have a carcinogenic effect possibly through estrogen adduct-mediated pathway and could further explain the link between this parasite and squamous cell carcinoma of the bladder.

The knowledge that *Schistosoma haematobium* has estrogenic molecules that are antagonists of estradiol may drive new therapeutic targets for this disease. The finding that these estrogens are cathecol estrogens with the ability to interact with DNA forming DNA adducts, could finally explain the link between schistosomiasis and bladder cancer. Therefore, the results obtained in this thesis may open potential new strategies for cancer treatment by targeting these estrogens, at least in schistosomiasis-associated bladder cancer.

FUTURE STUDIES

During the development of the present work, several questions were raised and these should be addressed in future studies:

- The results of this work were achieved by the use of Sh, which is made from parasite worms. The experiments described in the present work should be repeated with ova-derived antigens.

- The experiments addressing the carcinogenic-like phenotypes observed in CHO cells should be repeated using HCV29 cells.

- We observed dysplasia in the bladders of CD-1 mice instilled with Sh after 40 weeks. The time length of this animal experiment should be increased to search for higher grade bladder lesions.

- We found *KRAS* gene mutations in the dysplastic bladders. Other gene mutations should be looked for, like HRAS, which is the most common gene mutated in bladder cancer from the Ras family.

- Tumors of SCC of the bladder of schistosomiasis-infected individuals should be studied for *KRAS* and HRAS gene mutations as well as for estrogen receptor status.

- We identified four new estrogenic molecules from Sh. We will isolate and purify these estrogens, and repeat the *in vitro* and *in vitro* experiments described in this work, to study the effects of these compounds in normal cells in culture as well as in mice bladders. Estrogen receptor alpha and beta are downregulated by Sh. We will study the effect of Sh in the expression of the membrane-bound estrogen receptor GPR30 in MCF7 and HCV29 cell lines, mice bladders and tumors of bladder SCC.

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SUMMARY AND CONCLUSIONS

Since Fergusson (1911) epidemiological evidence indicates that *S. haematobium* is associated to squamous cell carcinoma of the bladder. However the mechanisms of this interaction are not clearly defined. Using normal epithelial cells, the purpose of the present work was to investigate whether or not *S.haematobium* parasite extracts were able to induce cancer-like phenotypes. To achieve this purpose we first used CHO cells to study proliferation, apoptosis, migration, invasion and tumorigenesis. We then used *in vivo* models to examine the effect of the parasite extracts on normal urothelium and assess its carcinogenic and mutagenic ability. To further elucidate the biological effects of this parasite, we identified in its extracts and in the sera of infected individuals new estrogenic molecules. The conclusions of our studies are as follows:

I - MECHANISMS UNDERLYING THE ASSOCIATION BETWEEN S. HAEMATOBIUM AND SQUAMOUS CELL CARCINOMA (SCC) OF THE BLADDER

First we obtained evidence that immature male worms of *S. haematobium* alone caused hepatic lesions in the golden hamster, a rare occurance, similar to those described for periovular granuloma. This study provided further experimental evidence for the role that schistosome worms, and their derived antigens, may play in the pathology of the infection of schistosomiasis, and prompted us to study the effects of *S. haematobium* total antigen (Sh) in normal cells. For this we used CHO cells and treated the cells in culture with Sh and found increased proliferation, decreased apoptosis, and induced migration and invasion. In addition, the parasite extract has also the potential to induce tumour development, we used the previous CHO cells exposed to Sh, after which they were inoculated subcutaneously into male nude mice.

These results encouraged us to investigate the effects of Sh in CD-1 mice normal bladders, after intravesical instillation of Sh. To further study specifically the carcinogenic alterations caused by Sh in the bladders of CD-1 mice, we looked for gene mutations in these bladders. Carcinoma of the bladder frequently harbors gene mutations that constitutively activate the receptor tyrosine kinase-Ras pathway and therefore we studied activating mutations in *KRAS* gene. According to our findings, intravesical instillation of Sh in CD-1 mice normal bladders, induced cell alterations in the urothelium consistent with dysplasia and inflammation. In the bladders of these animals, we also found that Sh has carcinogenic ability possibly through oncogenic mutation of *KRAS* gene.

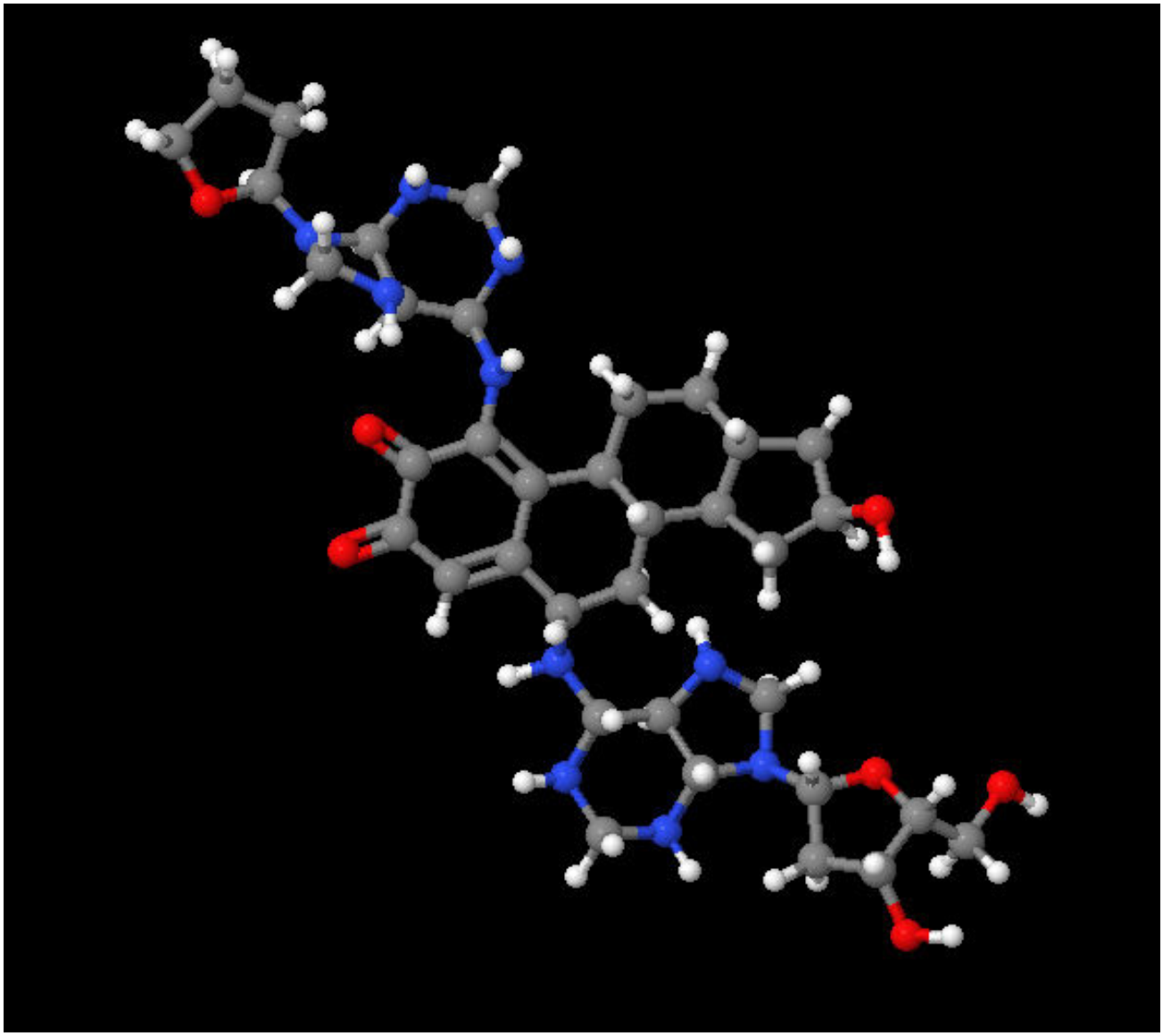
II - TO UNDERSTAND HOW HOST ENDOCRINE SYSTEM CAN FAVOR THE ESTABLISHMENT OF SCHISTOSOMES

While addressing schistosomiasis-induced hypogonadism by several hormones in patients infected with *S. haematobium* and *S. mansoni* we observed an extraordinary increase in serum levels of estradiol (E2). Surprisingly, the serum levels of the pituitary gonadotropins, Luteinizing Hormone (LH), and Follicle Stimulating Hormone (FSH), in these patients remained normal, excluding the normal regulating feedback system observed in the hypothalamic-pituitary-gonadal axis.

After identifying 4 new estrogens in Sh and in schistosomiasis-infected patients' sera by LC-ESI-MS, we tested the ability of these estrogenic molecules produced by *S. haematobium* to interact *in vitro* with Estrogen Receptor (ER) in the estrogenic responsive MCF7 cell line. Since we observed the repression of ER in MCF7 cells by Sh, we tested the hypothesis that Sh would also have the same effect in urothelial cells. Therefore we used HCV29 cells, a cell line of normal urothelial cells, and CD-1 mice to evaluate the expression of ER in the bladders of Sh instilled animals. Sh expresses estradiol-related molecules that down regulate ER alpha and beta in estrogen responsive cells and also in urothelial cells. These estrogens are also present

in the sera of *schistosoma*-infected patients, and they have the ability to repress ER transcriptional activity.

Based on our LC-ESI-MS results we propose the following structure for one of the estrogens, component 2 of the chromatogram, found in *S. haematobium* extracts and infected-patients' sera.



3D schematic representation of one of the estrogens found in *S. haematobium* total antigen and infected-individuals sera. The 3D representation allows us to infer that this molecule has the binding sites free to bind the receptor.

The estrogenic molecules present in *S. haematobium* extracts could have a carcinogenic effect possibly through estrogen adduct-mediated pathway and could further explain the link between this parasite and squamous cell carcinoma of the bladder.

SUMÁRIO E CONCLUSÕES

Desde Fergusson (1911) a associação entre infecção humana por *Schistosoma haematobium* e cancro da bexiga, particularmente o carcinoma espinocelular, tem sido sugerida, e é suportada por evidências epidemiológicas, estruturadas ou empíricas, nas áreas endémicas de schistosomose vesical. No entanto, nenhum mecanismo molecular da interacção foi descrito. Em busca de respostas a esta questão, o nosso propósito no presente programa, utilizando um modelo experimental de células epiteliais normais, procedemos no sentido de avaliar a capacidade do antigénio total de *S. haematobium* induzir, ou não, fenótipos celulares *cancer-like*. Inicialmente utilizamos células CHO (Chinese Hamster Ovary) para estudar mecanismos de proliferação celular, apoptose, migração e tumorigénese. De seguida, em modelos animais experimentais examinámos o efeito dos extractos antigénicos parasitários no urotélio e procedemos à avaliação da sua capacidade mutagénica e carcinogénica. Finalmente, e no sentido de construir uma hipótese plausível para compreender alguns efeitos biológicos do parasita averiguados nos nossos modelos experimentais, procedemos à identificação de novas moléculas de estrogénios, nos extractos antigénicos do parasita e no soro de doentes com schistosomose. Apontamos de seguida as seguintes conclusões deste trabalho:

I. *MECANISMOS IMPLICADOS NA ASSOCIAÇÃO ENTRE INFECÇÃO POR S. HAEMATOBIUM E CARCINOMA DAS CÉLULAS ESCAMOSAS DA BEXIGA.*

Subsequente a uma infecção murina experimental, observamos uma ocorrência rara: um granuloma celular envolvendo um verme adulto imaturo. Os componentes celulares deste granuloma eram em tudo semelhantes ao clássico granuloma periovular. Este facto sugere que componentes antigénicos do parasita (Sh) podem também eles desempenhar um papel

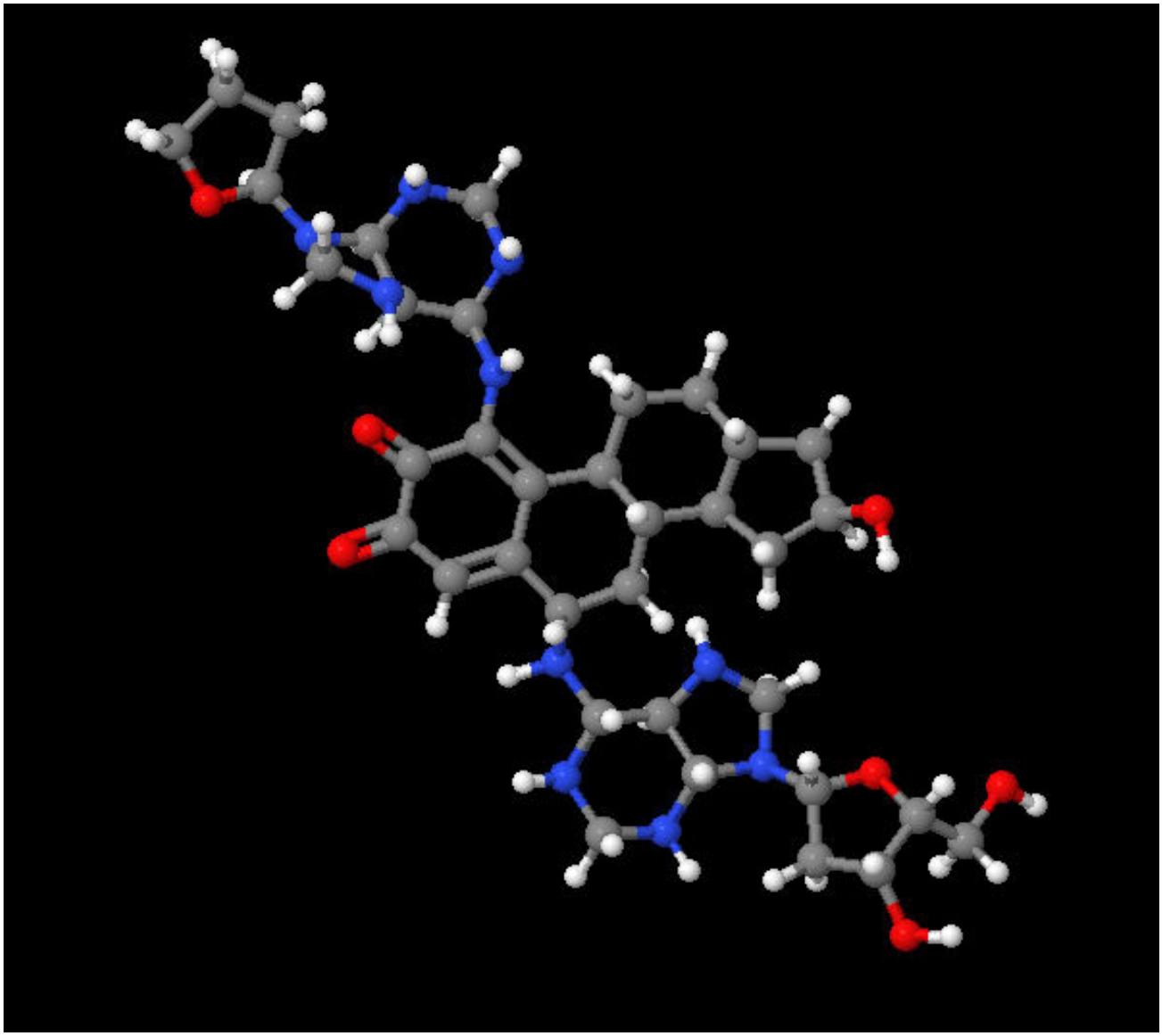
importante na patogenése da schistosomose. Esta evidência, em nosso entender, consubstancia a emergência dos modelos celulares experimentais propostos neste trabalho: utilização de Sh no estudo de efeitos biológicos em células normais. As células CHO em cultura e tratadas com Sh evidenciaram aumento da proliferação celular, apoptose diminuída e aumento da capacidade de migração e invasão. As mesmas células tratadas, após inoculação subcutânea num modelo murino atímico (nude mice), induziram o desenvolvimento de tumores.

O passo seguinte foi averiguar os efeitos do Sh no urotélio de um modelo murino normal (CD-1) após instilação intravesical. As bexigas dos animais assim tratados foram utilizadas para observação de alterações carcinogenicas e de mutações genéticas. De facto, o carcinoma da bexiga, na literatura, é frequentemente associado a mutações genéticas que activam o receptor da via Tirosina Kinase-RAS. Desta forma, decidimos observar as mutações no gene *KRAS*. A instilação de Sh nas bexigas de ratinhos CD-1 induziu alterações histológicas nos urotélios consistentes com displasia e inflamação e mutações oncogénicas no gene *KRAS*.

II. COMPREENDER O SISTEMA ENDÓCRINO DURANTE A INFECÇÃO.

A literatura é profícua na descrição de casos clínicos de hipogonadismo associado à infecção por *Schistosoma*. A busca de evidências para a compreensão destes casos permitiu-nos observar um dado novo: os níveis de estradiol estavam extraordinariamente aumentados nos doentes com schistosomose, independentemente do sexo ou idade. Nos mesmos doentes, os níveis normais das hormonas hipofisárias LH e FSH, pareciam excluir a atribuição de responsabilidade no evento ao sistema hipotalâmico-hipofisário-gonadal. Após análise por química fina de LC-ESI-MS foi possível proceder à identificação de 4 novas moléculas relacionadas com o estradiol nos soros dos doentes com schistosomose e no Sh. Por esta razão, decidimos observar o efeito do Sh e dos seus componentes estrogénicos, e particularmente a interacção com o Receptor de Estrogénio (RE), em células de cancro da mama MCF7, susceptíveis ao estrogénio. A repressão do RE nas células MCF7 tratadas com Sh foi significativa. Verificamos se ocorreria uma situação análoga nas células uroteliais dos

ratinhos CD-1 instilados intravesicalmente com Sh. Em células HCV29, uma linha de células uroteliais normais, tratadas com Sh bem como nas células uroteliais dos ratinhos CD-1 previamente descritos, procedemos à avaliação da expressão do RE. Nos dois casos experimentais a diminuição na expressão do RE, alfa e beta, era significativa. Demonstrámos a capacidade do Sh para frenar a actividade transcricional do RE, provavelmente, pela acção dos componentes estrogenicos constituintes. Propomos, como modelo de estrutura da molécula correspondente ao estrogénio mais abundante no cromatograma obtido em LC-ESI-MS, o seguinte:



Representação esquemática em 3D do estrogénio mais abundante encontrado no antigénio total de *S. haematobium* e no soro de indivíduos infectados. Esta representação em 3D permite inferir que a molecula mantém os locais de ligação ao receptor livres e que não enrola sobre si mesma.

As moléculas estrogénicas identificadas nos extractos de *S. haematobium* poderão ter um efeito carcinogénico, através da sua capacidade de provocar aductos no DNA. Este mecanismo poderá explicar a associação entre este parasita e o carcinoma espinocelular da bexiga.

PAPERS I-IX

PAPER I

RESEARCH PAPER

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Granulomatous-like immune reaction and hepatic fibrosis induced by *Schistosoma haematobium* immature worms

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Key words: *Schistosoma haematobium*, adult worms, liver lesions, liver fibrosis

Golden hamsters were inoculated with *Schistosoma haematobium* cercariae to examine histological lesions at different time points over an 18 month period of infection. Hamsters were sacrificed 26 weeks and 82 weeks after inoculation. The parasite was found in the blood and in the liver of infected animals as was expected, but we found exclusively male worms, no female worms nor eggs. Interestingly we observed unexpected hepatic lesions induced by *S. haematobium* adult male worms alone in the golden hamster, characteristic of schistosome eggs. Samples from liver, kidneys, lungs, bladder and gastrointestinal tract were collected during necropsy to evaluate injuries induced by *S. haematobium*. Notably we observed hepatitis in the liver of infected hamsters, no lesions were found in other organs. We also found liver fibrosis in infected hamsters. This study provides further experimental evidence for the role that schistosome worms, and their derived antigens, may play in the pathology of the infection and modulation of liver chronic inflammation in the murine model of schistosomiasis.

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Introduction

Human schistosomes currently infect more than 200 million people in 74 countries worldwide in the endemic areas of Africa, the Caribbean, Central America, South America, south-eastern Asia and the Middle East. Prevalence is thought to be rising mainly due to increasing travelers from the USA and Europe to these endemic regions for business or leisure. Wars are also known to increase the impact of schistosomiasis as demonstrated by a case recently published by our group.[1]

Of the three major human schistosome species, *S. haematobium*, causing urinary schistosomiasis, is the most prevalent species in sub-Saharan Africa where it is responsible for a substantial amount of schistosome-associated pathology.[2]

The histopathologic hallmark of an infection with schistosomes is periovular granuloma formation in the primary target organs, e.g., the liver. As the infection ages, important hepatic fibrosis develops, which in murine schistosomiasis is mainly egg granuloma-associated. Schistosomal granuloma formation is generally considered to be the result of a delayed-type hypersensitivity response generated by the host toward antigens secreted by tissue-deposited parasite eggs.[3-6] Schistosomal egg granuloma formation and ensuing hepatic fibrosis are intriguing pathophysiological processes demonstrating the complex interactions that exist between the host and the parasite. Parasite stage-specific factors as well as host organ-specific modalities and the genetic background have been identified or implicated in the pathogenesis of schistosomiasis.[6] However, experimental evidence previously obtained in the murine model of schistosomiasis mansoni also points toward the involvement of adult *S. mansoni* worms and their secreted antigens in the modulation of the egg antigen-induced liver granuloma and in the fibrotic response.[6-12]

In the present work, while studying schistosomiasis-associated inflammation, interestingly we observed unexpected hepatic lesions induced by *S. haematobium* immature male worms in the golden hamster. Here we characterized these lesions and the nature of the local immune response by examining the hepatic inflammatory infiltrate. We demonstrate the induction of hepatic fibrosis and hepatitis, characteristic of egg granulomas, induced by male adult worms of *S. haematobium* alone. This study provides further experimental evidence for the role that schistosome worms, and their derived antigens, may play in the pathology of the infection and modulation of chronic inflammation in the murine model of schistosomiasis.

Results

Animals' general aspect. During the experimental work no animal died and all, animals exhibited normal cage activity.

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Table 1. Challenge worm recoveries and antibody production analyzed by IHA

Group	Hamster	Weeks between challenge and perfusion	N° challenge cercariae	N° worm recovered	% recovery	IHA titre
Group1 (Control)	1	26	-	-	-	-
	2	26	-	-	-	-
Group 2 (Infected)	3	26	90	23	25.56%	1:160
	4	26	100	26	26.00%	1:160
	5	26	150	41	27.33%	1:320
Group 3 (Control)	6	82	-	-	-	-
	7	82	-	-	-	-
Group 4 (Infected)	8	82	90	24	26.67%	1:160
	9	82	100	28	28.00%	1:160
	10	82	95	25	26.32%	1:160

Table 2. Classification of histopathological data observed in liver sections obtained from hamsters infected with *Schistosoma haematobium*

Group	Hamster	P	PH	F	LPI	BP	E	MT	R
Group1 (Control)	1	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-
Group 2 (Infected)	3	+	++	+++	++	++	++	++	++
	4	+	++	++	++	+++	+++	++	++
	5	+	+++	+++	+++	+++	+++	+++	+++
Group 3 (Control)	6	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-
Group 4 (Infected)	8	+	++	++	++	++	++	+	+
	9	+	+++	+++	+++	++	++	+++	+++
	10	+	++	+++	+++	++	++	+++	+++

P, *S. haematobium* in histological section; PH, fibrous perihepatitis; F, lymphoid follicles; LPI, infiltration of lymphocytes and plasma cells; BP, bilharzial pigment; E, infiltration of eosinophils; MT, Masson Trichrome stain; R, Reticulin stain; -, absent; +, mild; ++, moderate; +++, severe.

The weight of the animals remained normal throughout the experiment.

Liver and fecal analysis, urinalysis, total adult worm burden and antibody production. Because Vuong et al.[13] reported that a mice *Mus musculus* infected with *S. haematobium* presented a squamous cell carcinoma of the urinary bladder, suggesting that the eggs from *S. haematobium* must cross the urothelium, while hamsters Mesocricetus displayed diverse lesions in digestive and genital tracts, liver and lungs, we searched the urine content, as well as the fecal content, for eggs to assert the single sex infection with males. All animals had their liver homogenate, feces and urine sediment negative for the presence of eggs as expected. In a time course of infections with *S. haematobium*, worm recovery was similar and remained constant in all groups. All worms were male. The antibody production was analyzed by IHA and found positive in all infected animals (**Table 1**).

Gross lesions. The livers from control groups showed no gross lesions on either the diaphragmatic or the visceral surface. Hamsters from groups 2 and 4 showed several tortuous whitish tracts. No macroscopic changes were observed in other organs. The weight of the organ remained normal throughout the experiment.

Microscopic lesions. No microscopic changes were seen in the spleen, lung, kidney, urinary bladder and gastrointestinal tract in samples obtained from all animals.

No liver histopathological changes were observed in hamsters from control groups. The liver histopathological findings observed in all groups are given in **Table 2**. Liver histopathology of animals infected with *S. haematobium* was characterized by dilated portal vein and marked periportal fibrosis (**Fig. 3B and D**). The number of liver flukes within dilated portal vein varies markedly and we did not observed any egg. **Figure 1B** shows a transverse section through a flat trematode worm within a dilated portal vein. Intestinal canal lined by simple columnar epithelium and viteline glands are seen.

Immunological worm reaction. Periworm granuloma-like formation occurred in all infected animals. The worms were lodged in portal veins of the liver (**Fig. 1B**). Granulomas of schistosome-infected hamsters are composed of numerous macrophages, eosinophilic granulocytes, lymphocytes and fibroblasts. Similar dominant cell types were characterizing the granulomatous-like reaction around the worms, thus closely resembling the hepatic granulomas induced by schistosome eggs although without the concentric layers (**Fig. 1B–D**).

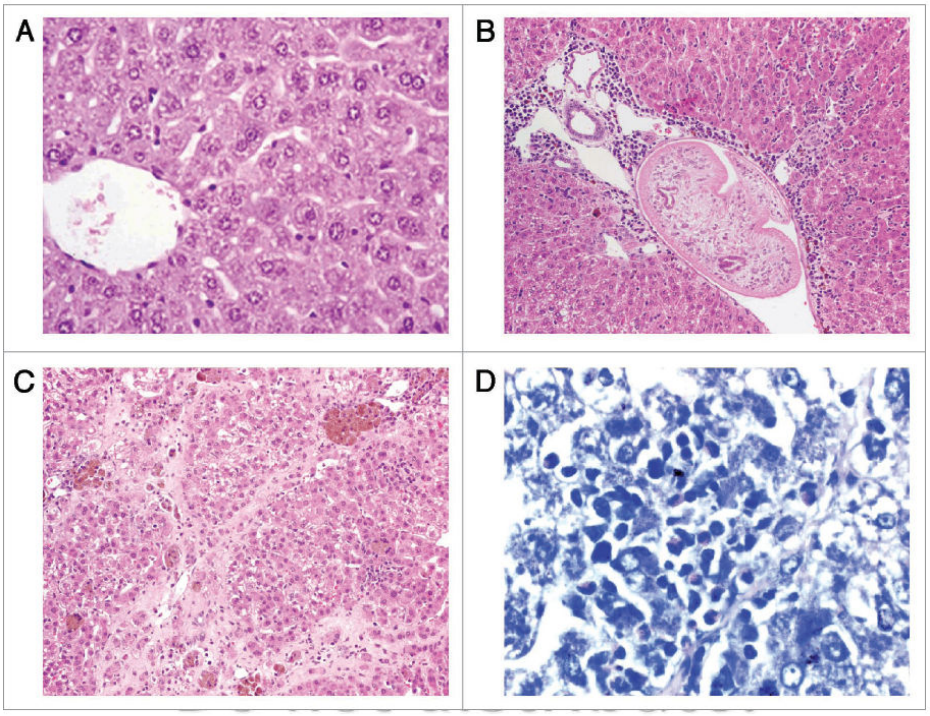


Figure 1. Histopathology of parasitic liver. (A) Control; (B) Liver section of *Schistosoma haematobium*-infected hamster, H&E, 400X; (C) Macrophages with abundant bilharzial pigment, H&E, 200X; (D) Eosinophiles infiltrate, Wright, 1000X.

Macrophages, fibroblasts and lymphocytes, and eosinophils were found to infiltrate along the outer layer of the hepatic induced granuloma-like. These histopathology appearances represent an early advanced feature of granuloma formation. Cellular infiltration was very apparent in the livers of infected animals. A slight to moderate inflammation was seen both within centrolobular vein and within portal areas (lymphocytes, plasma cells and macrophages with abundant bilharzial pigment) (**Fig. 1C**). Lymphoid follicles were often present in portal areas. These lesions were more severe in hamsters from group 4 than in those of group 2. Diffuse infiltration of eosinophils was often observed in areas of hepatic parenchyma and portal spaces (**Fig. 1D**).

The granules observed in the interior of macrophages were negative for hemosiderosis, bile granules or other type of infection as observed by the negative staining of PAS, Prussian Blue, Fouchet and Ziehl-Nielsen (**Fig. 2**).

Hepatic fibrosis. Schistosomiasis lead to a pathobiochemical reaction termed liver fibrosis. Thus, histological examination of a liver biopsy is essential for a diagnosis of liver fibrosis. All infected animals showed well-developed fibrosis. **Figure 3A** represents normal liver Masson Trichrome stain. The liver section showed in **Figure 3B** represents liver fibrosis, with portal-portal fibrous bridging and fibrous septa, connecting portal areas to each other's and lobule centers, hepatocytes were separated by blue colored collagen sheath. The aminopeptide of type III procollagen is the most widely used parameter. The reticulin technique was used to identify procollagen type III. In **Figure 3C** we may observe the normal trabecular pattern in the hamster liver sections stained for reticulin, which outlines the walls of sinusoids. Plates of hepatocytes in the normal adult liver are mostly one cell thick. In animals infected with *S. haematobium* we observe thin fibrous septa which tend to from bridges between damaged centrolobular zones (**Fig. 3D**).

Discussion

This study produced a very surprising finding: the consistent development of granulomatous-like immune reaction and fibrosis caused by adult worms of *S. haematobium,* characteristic of schistosome eggs. This detailed morphological study of hepatic lesions that occur in the hamster model of schistosomiasis is a contribution to understanding the pathogenesis of hepatic schistosomiasis. In the present work histopathological and histochemical studies were performed on the different organs. Histopathology

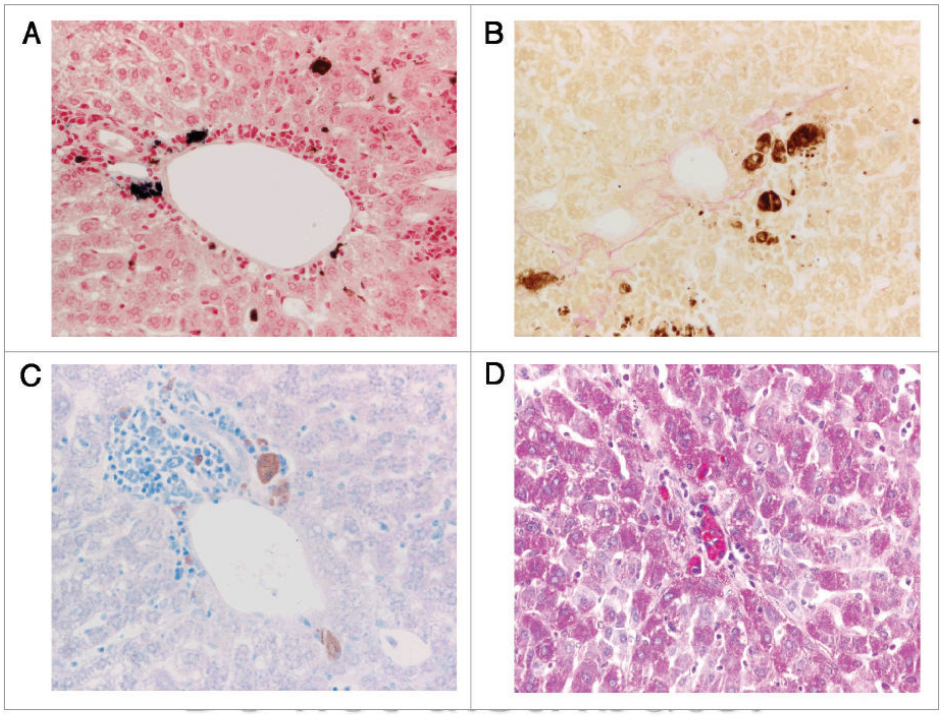
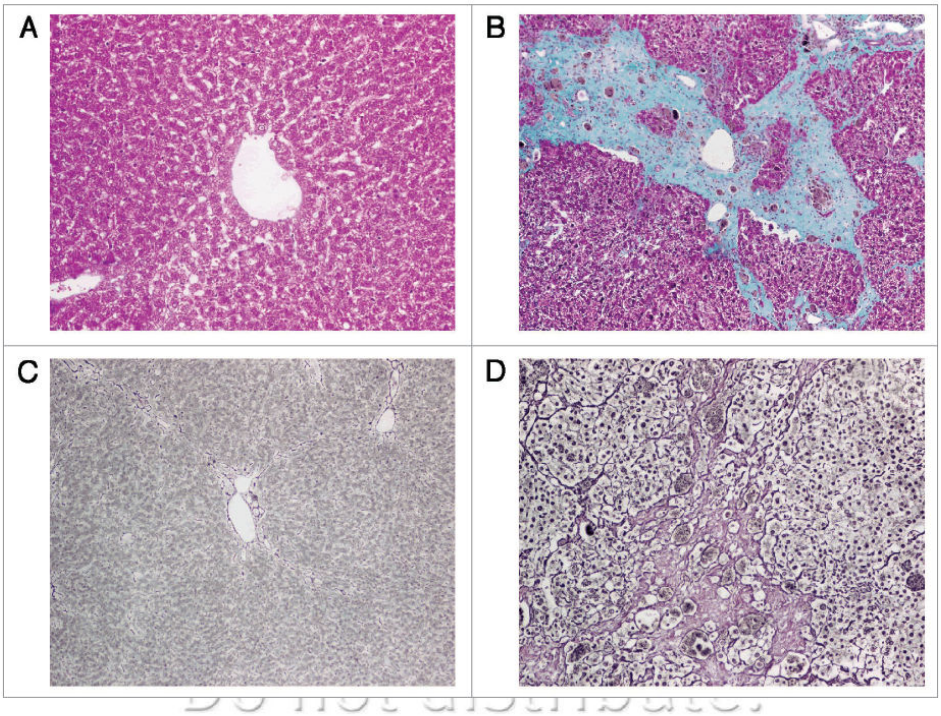


Figure 2. Macrophage granules. (A) Prussian blue, 400X; (B) Fouchet, 400X; (C) Ziehl-Nielsen, 400X; (D) PAS, 400X.

of liver sections from all infected hamsters revealed the presence of *S. haematobium* adult worms and fibrous perihepatitis. In addition, we recorded infiltration of eosinophils, plasma cells, macrophages with bilharzial pigment and fibroblasts. These features are the characterization of granulomas. Because these features, fibrosis and multinuclear cell formation are both hallmarks of granuloma development.[14] Our experimental single sex infection model revealed that adult worms had striking effects of granuloma-like development characterized by cellularity and/or cell composition (monocytes/macrophages, neutrophils and eosinophils) and fibrosis. In this experimental work, we did not observe *S. haematobium* eggs, this is explained by the inexistence of females during the experimental infection.

We have previously demonstrated that *S. haematobium* worm extract (Sh) has the potential to induce tumor development in a xenograft model, in which Sh-treated Chinese Hamster Ovary (CHO) cells formed tumors with similar phenotypes in all inoculated nude mice.[15] We have also demonstrated that Sh is likely to participate in a number of carcinogenesis mediated processes, such as increased cell proliferation and loss of p27, decreased apoptosis and increased expression of Bcl-2, and increased migration and invasion, all of which are processes needed for cancer cell survival,[16] as well as inducing dysplasia, a low grade intraepithelial neoplasia, in the bladders of CD-1 mice instilled with Sh.[17] Although we do not yet fully understand the host-parasite interactions of *S. haematobium* adult worms, or the immunological mechanism in associated fibrosis, the present study revealed intriguing novel aspects. There is no report in the literature that associates *S. haematobium* adult worms to hepatic fibrosis. But in schistosomiasis mansoni Baki et al.[18] reported hepatic fibrosis and histopathological lesions in mice experimentally infected with male *Schistosoma mansoni*. These authors revealed that, from the 25th week post-infection, a diffuse fibrosis affected the main branches of the portal vascular system following the host inflammatory reaction, associated with the proliferation of myofibroblasts in situ.[19] An increase of fibrotic deposit occurred during chronic unisexual infection suggesting that antigenic substances secreted by adult schistosomes, in the absence of any eggs, might initiate periportal and perisinusoidal fibrous reaction, confirming the results in the present paper.[19] It is generally accepted that the main lesions in established and chronic infection are due not to the adult worms but to eggs that are trapped in the tissues. The eggs secrete proteolytic enzymes that provoke typical eosinophilic inflammatory and granulomatous reactions, which are progressively replaced by fibrotic deposits.[20] It is intriguing that the worms caused the alterations described above in liver



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Figure 3. Liver fibrosis (A) Hamster liver section with normal Masson Trichrome stain, Trichrome stain, 200X; (B) Portal-portal fibrous bridging and fibrous septa, connecting portal areas to each others and lobule centers, Masson's trichrome stain was used. Collagen fibres show blue staining. Magnification 400X. (C) Normal reticulin pattern, reticulin stain, 100X; (D) Black lines between hepatocytes, hamster with hepatic fibrosis, reticulin stain, 200X.

tissue similar to the ones described in egg granuloma-associated fibrosis. The most studied feature in the pathology of schistosomiasis is currently the immunogenicity of the egg antigens. Schistosome eggs express various glycosylated proteins and lipids that are able to induce humoral and cellular immune reactions.[21] Nevertheless, schistosome worms and eggs antigens share common molecules.[21,22]

We offer one possible explanation for the worm-induced granuloma-like and fibrosis in our model. In the course of an infection, it is established that the immune response progresses through at least three phases. In the first 3–5 weeks, during which the host is exposed to migrating immature parasites, the dominant response is T helper 1 (Th1)-like. As the parasites mature, mate and begin to produce eggs at weeks 5–6, the response alters markedly; the Th1 component decreases and this is associated with the emergence of a strong Th2 response. Immature dendritic cells (DCs) can acquire schistosome egg antigens and induce Th2 responses, but the process by which this occurs is unclear. Primarily, this response is induced by egg antigens.[23] It is possible that in the absence of eggs DCs acquire schistosome worm antigens instead and induce also a Th2 response. In accordance with our explanation it is known that established worms elicit an immune response which prevents continuous further accumulation of worms while themselves remaining invulnerable to immunological attack.[24] In accordance we found positive immune reaction detected by IHA in all infected animals.

In contrast, Moloney and collaborators,[25] demonstrated that schistosome female worms from single sex infections induced no overt pathology in the host. Male worm burdens from these single sex infections induced distended hepatic portal veins and marked deposition of pigment in the livers of infected mice but induced neither hepatic lesions nor immune cell infiltrate. On the other hand, in agreement with our work, Jacobs et al.[6] demonstrated the positive modulation of hepatic *S. mansoni* egg antigen-induced granuloma formation and periparticulate fibrosis by living adult *S. haematobium* worms, resulting in more severe liver pathology. These researchers demonstrated the complex interactions that exist between parasite and host. Once more, the implication of the schistosome worm as a *primum movens* in the genesis and modulation of fibrosis has been demonstrated and therefore Schistosome worms deserve our full attention in the unraveling of the mechanisms underlying schistosome infection and pathology.[6]

The similarity in immune response seen in mice infected with *S. haematobium* worms compared to the immune response caused by eggs might provide important clues regarding the nature of the disease. While the use of egg-induced granulomas are the most common model to study fibrosis, to the best of our knowledge, experimental data using worms to study hepatic fibrosis have not been published. In the present study we employed single sex adult worms' infection of *S. haematobium* to clarify the stage specific roles of adult worms. Specifically, we looked for similarities of *S. haematobium* adult worm induced hepatic granuloma-like development and known egg-induced granuloma. The results of this study strongly indicate crucial roles for adult worms of *S. haematobium* in chronic stage of hepatic granuloma formation, especially in regards to fibrosis and the inflammatory cell types present.

In this in vivo study we demonstrated the positive modulation of hepatic fibrosis and hepatitis by living adult *S. haematobium* worms alone. These results reinforce the applicability of hamsters as a suitable model of fibrosis and hepatitis and provide new insights into the response of experimental models to *Schistosoma haematobium* infection.

Material and Methods

Animals. Eight-week-old female golden hamsters (LVG/SYR) were provided by Charles River (Barcelona, Spain). Animals spent one week being acclimated under routine laboratory conditions before starting the experiments. They did not receive any treatment prior to the study. Hamsters were kept in separated cages and fed standard balanced food and water ad libitum. All the animals were raised and 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).

Parasites. *S. haematobium* (Angolan strain) life cycle was maintained through successive passages in laboratory-raised *Bulinus truncatus* as invertebrate hosts and golden hamsters as vertebrate hosts. Cercariae of *S. haematobium* were obtained from infected snails by the use of artificial light.

Experimental infection. Ten hamsters were divided into 4 groups. Groups 1 and 3 (n = 2) were control groups. Groups 2 (n = 3) and 4 (n = 3) were infected with *S. haematobium*. Groups 1 and 2 were sacrificed 26 weeks after infection; group 3 and 4 were sacrificed 82 weeks after infection. Hamsters were experimentally infected by transcutaneous route with approximately 100 cercariae of *S. haematobium*. For the duration of the study, the hamster's state of health was monitored daily.

Animal's liver, stool and urine analysis. For the stool and urine collection animals were kept in single mouse metabolic cages (Tecniplast, Buguggiate, Italy) for 24 hours. The animals were acclimatized for 12 hours per day, 2 days before the 24 hours assay. This cage allows absolute and immediate separation of feces and urine. After the 24 hours assay feces and urine was collected and analyzed for the presence of eggs. The livers were removed from freshly killed mice. The organs were digested in 4% potassium hydroxide at 56°C and centrifuged (900 g) for 5 min. For oogram performance, duplicate 100 µL aliquots of the digest were placed on a glass slide and eggs were searched by light microscopy (100X).

Serum antibody determination by indirect haemagglutination (IHA). Sera were obtained from blood samples collected from infected animals at euthanasia. Mice were individually tested for the quantitative detection of the antibodies present in their sera. Determination of antibodies specific for Schistosoma was performed as previously described.[27] The IHA schistosomiasis kit obtained from Fumouze Laboratories (Levallois-Perret, France) was used according to the instructions of the manufacturer. Briefly, the test procedure was as follows. Fifty microliters of a 1:20 initial dilution of each serum was subjected to further twofold serial dilutions, and 10 µl of sheep red blood cells sensitized with *S. mansoni* immature worm antigens was added to each diluted sample. Positive and negative control sera and nonsensitized red blood cells were included in each test as controls for naturally occurring antibodies. After incubation for 2 h at room temperature the titer in the test serum was recorded as one dilution before that which yielded a clear, sharp dark spot similar to those in the negative control wells. Titers were expressed as reciprocal values. All sera were tested in duplicate. The results were evaluated with a cutoff titer of 1:160 as recommended by the manufacturer.

Total immature worm recovery. The immature worm burden was determined by total blood perfusion and the numbers of worms determined. Immature worms of *S. haematobium* were recovered at 26 and 82 weeks after challenge by perfusion of the hepatic portal system via the aorta with citrated saline. Hamsters were perfused under anesthesia through the heart with citrated phosphate-buffered saline and the worms were recovered. Worm burdens were estimated after portal perfusion through an incised portal vein of infected mice euthanized by an anesthetic overdose.

Macroscopy. At the time of perfusion, complete necropsies were carefully conducted. After opening the abdomen, all organs were examined macroscopically for any changes; the organs were collected, weighed and immersed in 10% phosphate buffered formalin. Representative fragments of all organs were fixed in buffered formalin 10%. Tissue sections (2 µm) were stained with each of the following: haematoxylin and eosin (HE); Wright for eosinophiles, Ziehl-Nielsen, PAS, Fouchet and Prussian Blue for macrophage granules and reticulin and Masson trichrome for collagen.

Masson's trichrome. Masson's trichrome staining protocol is used to stain collagen fibers. Liver sections were deparaffinized and rehydrated through 100% alcohol, 95% alcohol, 70% alcohol. Then were washed in distilled water and stained in Weigert's iron haematoxylin working solution for 10 minutes. After sections were rinsed in running warm tap water for 10 minutes and washed in distilled water. Sections were after stained in Biebrich scarlet-acid fuchsin solution for 15 minutes and washed in

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distilled water, differentiated in phosphomolybdic-phosphotungstic acid solution for 15 minutes. Sections were then transferred directly (without rinse) to aniline blue solution and stained for 5–10 minutes. Rinsed briefly in distilled water and differentiated in 1% acetic acid solution for 2–5 minutes. Later were washed in distilled water and dehydrated through 95% ethyl alcohol, absolute ethyl alcohol and clear in xylene. Finally were mounted with resinous mounting medium.

Reticulin. The reticulin technique was based on the following technique: liver sections were deparaffinized in xylene then took through alcohols to water. Sections were oxidized in acidified potassium permanganate during 3 minutes, rinsed in distilled water and decolorized with 2% oxalic acid for 1 minute. Sections were rinsed again in distilled water, placed in mordant in 4% iron aluminium for 10 minutes, and rinsed in distilled water. Sections were impregnated in ammoniacal silver solution for 11 seconds and quickly rinsed in distilled water. Sections were immediately reduced with 10% aqueous formalin for 2 minutes and washed in running tap water for 2 minutes. Sections were toned in 0.2% gold chloride for 2 minutes and rinsed in distilled water, fixed with 2% aqueous sodium thiosulphate (hypo) for 2 minutes and washed in tap water for 2 minutes. Sections were counterstained with neutral red for 2 minutes, dehydrated, cleared and mounted.

Histological analysis. A histological study was conducted on H&E-stained tissue sections to evaluate the following parameters: presence of *S. haematobium* immature worms in histological section; fibrous perihepatitis, lymphoid follicles, infiltration of lymphocytes and plasma cells; and the presence of bilharzial pigment. Wright-stained tissue sections were used to evaluate infiltration of eosinophils. Masson Trichrome and Reticulin stains were evaluated. To assess the severity of hepatic lesions, two researchers evaluated tissue sections independently, as follows: -, absent; +, mild; ++, moderate; +++, severe.

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Schistosoma haematobium total antigen induces increased proliferation, migration and invasion, and decreases apoptosis of normal epithelial cells

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ABSTRACT

Schistosome worms are blood-dwelling flukes that cause chronic infection in more than 200 million people and are thought to be responsible for 500,000 deaths annually. During infection with *Schistosoma haematobium*, eggs are deposited in the mucosa and submucosa of the bladder and lower ureters. Squamous cell carcinoma (SCC) of the bladder is a long-term sequela of chronic infection. The mechanisms underlying the association between *S. haematobium* and SCC of the bladder are largely unknown, with all reports to date exclusively demonstrating epidemiological evidence linking *S. haematobium* infection with SCC of the bladder. We hypothesised that the parasite antigens might induce alterations in epithelial cells towards cancer. For this we used Chinese Hamster Ovary (CHO) cells and treated the cells in culture with *S. haematobium* total antigen (Sh). Our results showed increased proliferation, increased *S*-phase and decreased apoptosis, as well as down-regulation of tumour suppressor p27 and up-regulation of anti-apoptotic molecule Bcl-2 in Sh-treated cells compared with controls. We also found increased migration and invasion. To our knowledge, this is the first report demonstrating alterations of normal epithelial cells as a direct effect of *S. haematobium* antigens.

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1. Introduction

Current World Health Organization estimates indicate that over 600 million people are at risk of acquiring schistosomiasis (also known as Bilharziasis or snail fever), with approximately 200 million infected worldwide, mainly in rural agricultural and peri-urban areas. Of these, 20 million suffer major consequences from the disease and 120 million are asymptomatic. In the developing world, parasitic infections such as schistosomiasis are common, recurrent and long-lasting health problems that represent an ongoing inflammatory challenge and a significant health threat to the populations who are exposed to continuing daily risk of infection (King and Dangerfield-Cha, 2008; Hotez et al., 2008).

In Egypt and in some other regions of the Middle East and Africa, bladder cancer accounts for approximately 30% of all cancers, where it is the most common malignancy in males and the second most common in females after breast cancer (El-Sheikh et al., 2001). This high incidence of bladder cancer is strongly related to the endemic parasite *Schistosoma haematobium*. Most investigators have accepted the association between schistosomiasis and bladder cancer since the work of Ferguson (1911). Evidence for this relationship derives from the geographical correlation between the conditions, distinctive patterns of gender and age at diagnosis, clinicopathological features of *Schistosoma*-associated bladder cancer and evidence obtained in experimental models (El-Sheikh et al., 2001). The mechanisms underlying the association between *S. haematobium* and squamous cell carcinoma (SCC) of the bladder are largely unknown, with all reports to date exclusively demonstrating epidemiological evidence linking *S. haematobium* infection with SCC of the bladder.

We have previously documented evidence for the oncogenic potential of *S. haematobium* total antigen by its treatment of Chinese hamster ovary (CHO) cells resulting in increased proliferation and tumourigenesis in nude mice (Botelho et al., 2009). In the present work we adapted in vitro models of proliferation, apoptosis and invasion previously used to study tumour cells, to study the effects of *S. haematobium* total antigen (Sh) in normal mammalian cells.

Most cancer can be considered as disorders of differentiation with uncontrolled self-renewal of undifferentiated cells resulting in an imbalance between cell proliferation and programmed cell

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death (Miyake et al., 2001). We analysed several cancer related phenotypes such as cell proliferation, apoptosis, migration and invasion in order to evaluate the oncogenic potential of Sh through biochemical and functional studies to characterise the effects of Sh in CHO cells. We show that it is intimately involved in the regulation of proliferation by decreasing tumour suppressor p27, apoptosis by up-regulating anti-apoptotic molecule Bcl-2, motility and invasion.

2. Materials and methods

2.1. Patients

Urine was collected from *S. haematobium*-infected individuals. The individuals were living in Angola, an endemic area for schistosomiasis. Following instruction in midstream urine collection, urine samples were collected from each individual. *Schistosoma haematobium* infection was detected by microscopic observation of eggs in the sediment of centrifuged urine. Informed consent was obtained from individuals. The eggs were hatched and snails from susceptible species were infected with the resulting miracidia (Viana da Costa et al., 1998).

2.2. Animals and experimental infections

As described previously, golden hamsters and BALB/c mice were experimentally infected with 100 cercariae by tail immersion. The control animals consisted of littermates. The cercariae were obtained by shedding of snails infected with miracidia (Gaubert et al., 1999). The experiments were carried out 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).

2.3. Schistosoma haematobium total antigen production (Sh)

Angolan strain *S. haematobium* adult worms were collected by perfusion of the hepatic portal system of golden hamsters 7 weeks p.i. with 100 cercarie. The worms were suspended in PBS and then sonicated. The protein extract was then ultracentrifuged and the protein concentration was estimated using a micro BCA protein assay reagent kit (Viana da Costa et al., 1998).

2.4. Cell lines

CHO cells were cultured and maintained at 37 °C in a 5% CO_2 humidified atmosphere in CHO medium (Sigma) with 10% FBS and 1% penicillin/streptomycin (Sigma). Cells were passaged every 5 days. Before treatments cells were serum-starved for 16 h.

2.5. Trypan blue exclusion assay

The trypan blue exclusion method was used to assess cell viability. CHO cells were plated onto 24-well plates at approximately 1.0×10^4 cells per well and incubated for 24 h. The cells were treated with Sh at 50 μg/ml for 24 h. On days 0–4 after treatment, the cells were harvested by trypsinization and counted under a microscope after trypan blue staining. Three independent experiments were carried out based on the following formula: cell viability % = number of cells in drug treatment group/number of cells in control group × 100%.

2.6. Proliferation assay

The CellTiter 96 AQ non-radioactive cell proliferation assay (Promega) was used to assess cell viability. The assay is composed of the tetrazolium compound MTS and an electron coupling reagent, PMS. MTS is reduced by viable cells to formazan, which can be measured with a spectrophotometer based on the amount of absorbance at 490 nm. Formazan production is time-dependent and proportional to the number of viable cells. CHO cells were cultured in 0.1 ml CHO media in 96-well flat-bottomed plates. Cultures were seeded at 1×10^4 cells/well and allowed to attach overnight. After the indicated time of incubation with the appropriate medium, 20 μl reagent was added per well, and cells were incubated for 1 h before measuring absorbance at 490 nm. Background absorbance from the control wells was subtracted. Studies were performed in triplicate for each experimental condition (Soares et al., 2007).

2.7. Cell sample preparation and histochemistry

Samples of Sh-treated and control CHO cells were collected. After centrifugation, cell sediments were used to prepare smears. For morphological studies, cytospin smears were routinely fixed and stained using H & E. An aliquot of each specimen was centrifuged onto a cytospin slide (Cytoslide™; Thermo Electron Corporation) by rotating in a Shandon Cytoslide™ device at 800 rpm. for 5 min. The smears resulted in a single layer of cells. At least three cytospins were prepared from each specimen. H & E stains were performed according to standard protocols.

2.8. Cell cycle assay

After treatments described above, cells (1.5×10^5/L cells) were suspended in medium, washed once with PBS, lyzed and stained with propidium iodide (PI) at room temperature for 15 min. DNA histograms were assayed by flow cytometry (Becton–Dickinson). In each sample, a minimum of 10,000 cells was counted and stored in list mode. Data analysis was performed using standard Cell Quest software (Becton–Dickinson).

2.9. Western blot analysis

Western blot analyses of tumour suppressor p27 were performed in Sh-treated cells and controls with an anti-p27 monoclonal antibody and normalised with β-actin expression (Santa Cruz Biotechnology, Santa Cruz, CA, USA), detected by a chemiluminescent method and the resulting bands evaluated by an imaging densitometer (BioRad GS-670). Experiments were repeated at least twice.

2.10. Apoptosis

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick endlabeling (TUNEL) assay was performed using the in situ cell death detection kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. Nuclei were counter-stained with DAPI (Roche Diagnostics, Basel, Switzerland). The percentage of TUNEL-stained nuclei was evaluated in relation to every DAPI-stained nuclei observed. Immunofluorescence was visualised under a fluorescence microscope (Olympus, BH-2, UK). The percentage of stained cells was evaluated by counting the cells stained with TUNEL, divided by the total number of nuclei stained with DAPI at a magnification 200× field. One thousand nuclei were evaluated. Three independent experiments were performed. Apoptosis in cell cultures was assessed with the in situ cell death detection kit, fluorescein (TUNEL technology) (Roche), analysed by fluorescence microscopy (Soares et al., 2007).

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2.11. Bcl-2 expression analysis

The monoclonal antibody against Bcl-2 was used (Dako A/S, Glostrup, Denmark) conjugated with FITC. Briefly, 2×10^5 cells were first permeabilized and fixed using a Fix and Perm kit (CALTAG, San Francisco, CA, USA), and then the cells were stained with Bcl-2/FITC. Data acquisition and analysis were performed by FACScalibur flow cytometer (Becton–Dickinson) (Arden et al., 2007).

2.12. Cell migration

The in vitro wound closure assay was performed to analyse cell migration. Cells were seeded on six-well plates in triplicates and grown to 90% confluence. Then the cell monolayers were scratched with a pipette tip. Three scratches were photographed with a digital compact camera immediately after wounding and then every 24 h for 72 h (Suriano et al., 2003).

2.13. Cell invasion

Cell invasion was analysed in 24-well Matrigel-coated invasion chambers (BD Biosciences, Bedford, MA) according to the manufacturer's directions with the following modifications: hydrated chambers were transferred to a new 24-well plate containing medium with 20% serum. Cells (5×10^5; treated with Sh or control) were added to each chamber and incubated for 20 h. Cells that did not pass through the filter were removed with a cotton swab prior to processing the filter with a Hema-3 staining kit (Fisher, Pasadena, CA, USA) and mounting the filter on a microscope slide. Cells in five fields on the filter were photographed at 40× magnification and counted manually for each treatment (Soares et al., 2007).

2.14. Statistical analysis

Data were expressed as mean ± S.D. Student's *t* test was used to assess the statistical significance of differences. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Sh total antigen increased the proliferation of epithelial cells in vitro

Cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis (Hanahan and Weinberg, 2000). To begin investigating the effect of Sh on cell viability and proliferation, CHO cells were seeded on 96-well plates, starved overnight, treated with increasing concentrations of Sh for 24 h, cultivated for 24, 48 and 72 h and then analysed by MTS assay (Fig. 1A). The growth curve shows that treated cells proliferated significantly faster and more than control cells. To evaluate the effect of Sh on cell viability we used a trypan blue exclusion assay. Cell numbers were assessed by direct daily counting (Fig. 1B). We

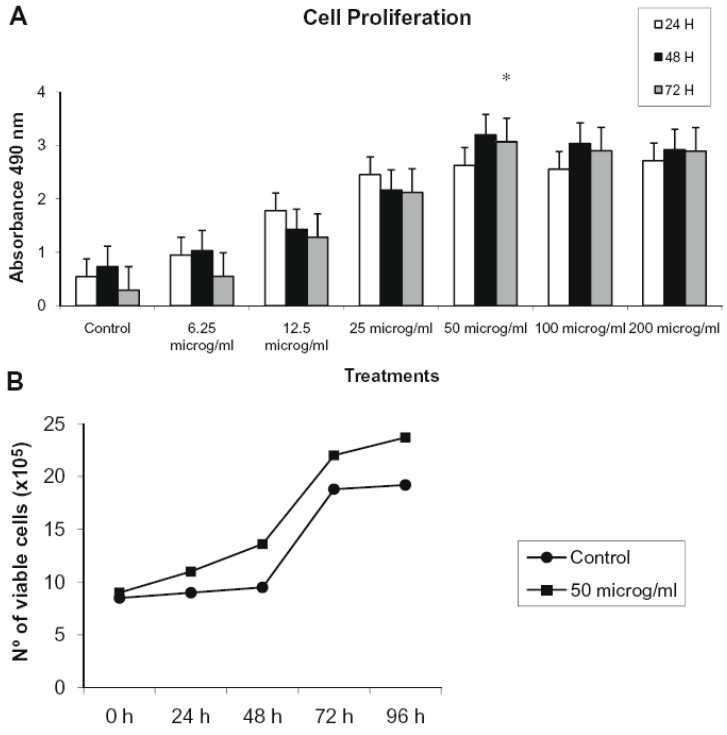


Fig. 1. Cell proliferation assay of *Schistosoma haematobium* total antigen (Sh)-treated cells. (A) The growth curve shows that treated cells proliferated significantly faster and more than control cells ($P < 0.01$; Control versus 50 μg/ml). (B) Trypan exclusion assay of control and Sh-treated cells ($P < 0.01$; control versus Sh). We used the concentration of 50 μg/ml of Sh for the subsequent assays. We confirmed the increase in cell viability by trypan exclusion assay. Cell numbers were assessed by direct daily counting. The experiments were done in triplicate.

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confirmed the increase in cell viability using the concentration of 50 μg/ml of Sh. This concentration was used for the subsequent assays. Our results suggest that the increase of both proliferation and overall survival in CHO cells is a consequence of Sh treatment.

We hypothesised that increased cell proliferation is the result of cell cycle progression driven by Sh, and therefore analysed cell cycle progression with flow cytometry (Fig. 2 and Table 1). This method revealed that cell growth induction by Sh was the result of an increase in *G2/M*- and *S*-phase and a decrease of *G0/G1*-phase (Fig. 2B), compared with controls (Fig. 2A).

The cytospin followed by H & E staining of Sh-treated cells showed threefold more cells in mitose (Fig. 5E and F) than control cells (data not shown), confirming the proliferating advantage of Sh-treated cells.

We further examined whether Sh affects the expression of cell cycle-related proteins. Expression of p27, a cyclin dependent kinase inhibitor (CDKI) was analysed in CHO cells after Sh treatment. Immunoblotting for p27 was performed in CHO cells. p27 protein was down-regulated in Sh-treated CHO cells (Fig. 2C). Thus, *S. haematobium* alters the cell cycle of treated cells as demonstrated by down-regulation of CDKI p27 expression of normal epithelial cells in culture.

3.2. Sh decreased the apoptosis of epithelial cells

To analyse apoptosis, CHO cells were seeded on 96-well plates, starved overnight, treated with increasing concentrations of Sh for 24 h, cultivated for 24, 48 and 72 h and then analysed by TUNEL assay (Fig. 3). CHO cells showed an increasing number of apoptotic cells per field in the control (Fig. 3A) compared with cells treated with 50 μg/ml of Sh (Fig. 3B). Cell counts show that treated cells markedly decreased apoptosis compared with control cells (Fig. 3C).

Since the Bcl-2 family of proteins are regarded as key regulators of apoptosis, the influence of Sh on Bcl-2 expression levels in CHO cells was subsequently investigated. Bcl-2 is perceived as anti-apoptotic (Miyake et al., 2001). In this study Sh exposure led to significant increases in Bcl-2 expression levels compared with controls (Fig. 3D). The percentage of Bcl-2-positive cells in the control group (a) was 50% whereas in the Sh-treated group it was 53% (b).

3.3. Sh induces alterations in the morphology of treated cells

Serum deprivation is normally used to avoid unspecific effects of serum components in cellular behaviour. Therefore CHO cells were cultured in serum-free medium overnight. Interestingly, we observed unexpected morphological differences in CHO cells

Table 1
Effects of treatment with *Schistosoma haematobium* total antigen (Sh) on cell cycle distribution 48 h after treatment.

Groups	Cell cycle		
	G0/G1	*S*	*G2/M*
Control	80.61 ± 3.39	16.67 ± 2.73	2.72 ± 0.92
Sh-treated	78.07 ± 4.04	18.59 ± 3.42[a]	3.34 ± 0.97[a]

Data are expressed as mean ± SD of the three experiments.
[a] $P < 0.05$.

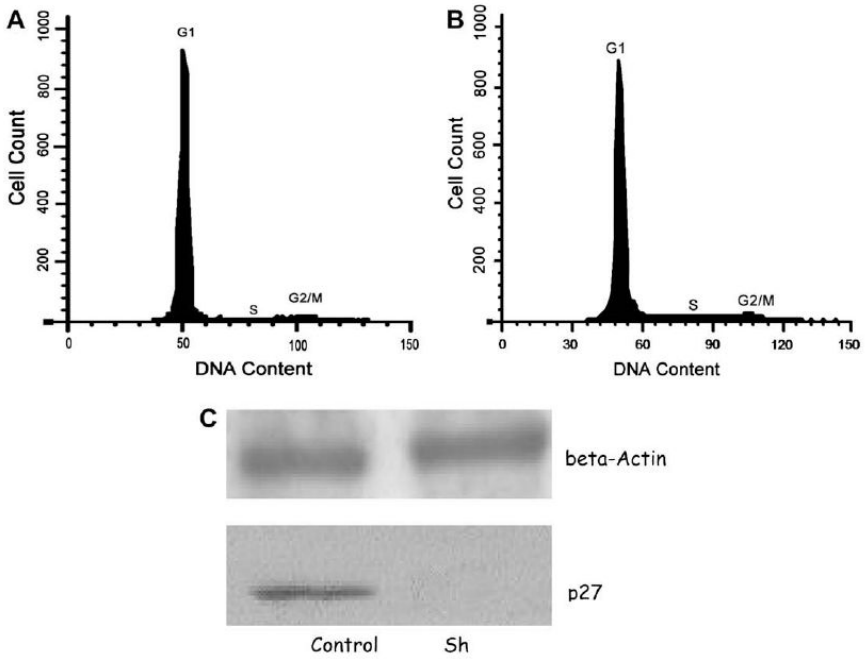


Fig. 2. Effects of treatment with *Schistosoma haematobium* total antigen (Sh) on cell cycle distribution 48 h after treatment. (A) Control cells. (B) Flow cytometry revealed that the cell growth induction by Sh was a result of an increase in *G2/M*- and *S*-phase and a decrease in *G0/G1*-phase, compared with controls. (C) Expression of p27, a cyclin-dependent kinase inhibitor was analysed in Chinese hamster ovary (CHO) cells after Sh treatment. Immunoblotting for p27 was performed in CHO cells. p27 protein was down-regulated in Sh-treated CHO cells.

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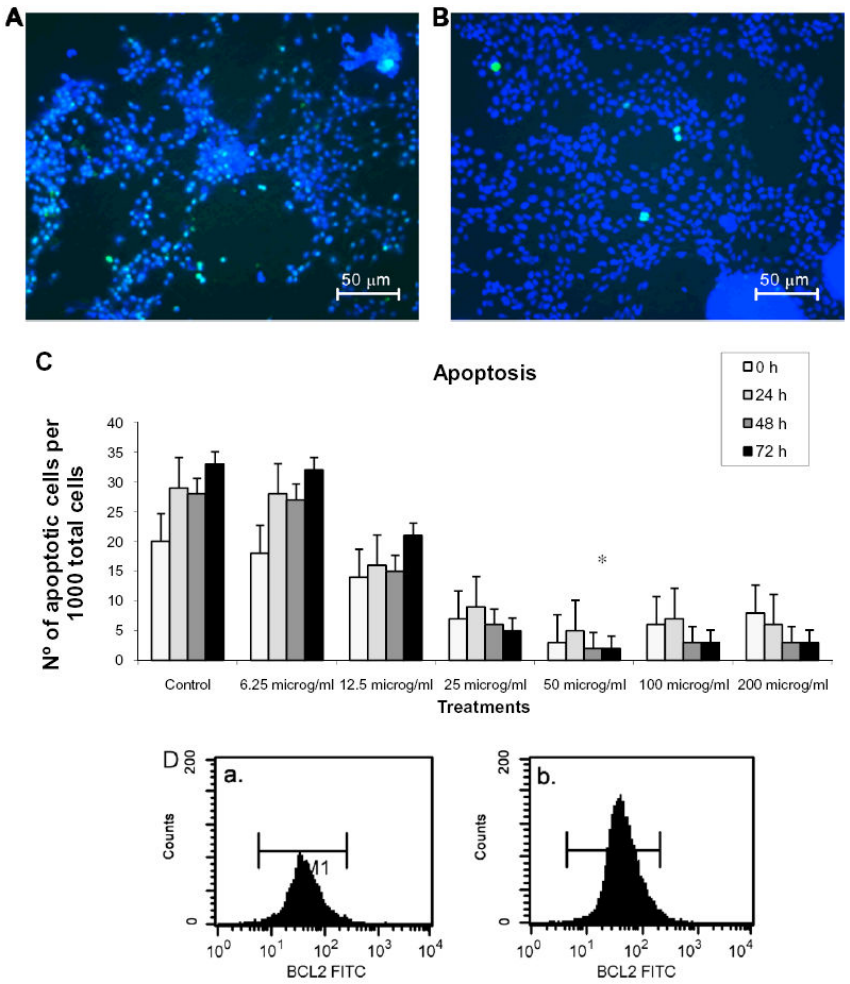


Fig. 3. Apoptosis of *Schistosoma haematobium* total antigen (Sh)-treated cells analysed using TUNEL. (A) Microphotographs of apoptotic cells of controls and (B) Sh-treated cells. Both panels have the same magnification. Chinese hamster ovary (CHO) cells, showing an increasing number of apoptotic cells per field in the control (A) compared with cells treated with 50 µg/ml of Sh (B). The white bars represent 50 µm. (C) The growth curve shows that treated cells markedly decreased apoptosis compared with control cells ($P < 0.01$; control versus 50 µg/ml). (D) Histogram of CHO cells expressing anti-apoptotic molecule Bcl-2 by fluorescence-activated cell sorting analysis. Control (a) and Sh-treated cells (b). In this study Sh exposure led to a significant increase over controls in Bcl-2 expression levels. The experiments were done in triplicate.

treated with Sh compared with controls, photographed by phase contrast microscopy (Fig. 4). Control cells show aberrant morphology induced by serum deprivation (Fig. 4A). In contrast, after serum deprivation cells treated with 50 µg/ml of Sh displayed polarised lamellipodia (Fig. 4B).

The morphological differences were further confirmed by H & E staining after cytospin. The cells treated with Sh are bigger, with larger nuclei and cytoplasms (Fig. 5B) than control cells (Fig. 5A). The cell membranes of treated cells (Fig. 5D) presented more integrity than control cells (Fig. 5C).

3.4. Induced migration and invasion of epithelial cells in vitro by Sh

Cell motility and tissue invasion were suggested to be two of the essential alterations in cell physiology that collectively dictate malignant growth (Hanahan and Weinberg, 2000). Cell migration was analysed with an in vitro wound closure assay (Fig. 6). In control cells, the ability to migrate was limited, with the wound remaining open after 48 h (Fig. 6E). In contrast, in Sh-treated cells, there are clear signs of cell migration after 24 h (Fig. 6D) with close to complete wound closure after 48 h (Fig. 6F).

To test whether Sh induces invasion in vitro, we measured invasion using Boyden chambers (Fig. 7). Membranes in the chambers were coated with Matrigel to mimic basement membrane. Movement of cells through the membrane required not only migration but also degradation of matrix, a critical step in the invasion process. Our results show that treatment of cells with Sh stimulated invasion (Fig. 7B) compared with controls (Fig. 7A). Sh induced the invasion of treated cells fivefold more than control cells (Fig. 7C).

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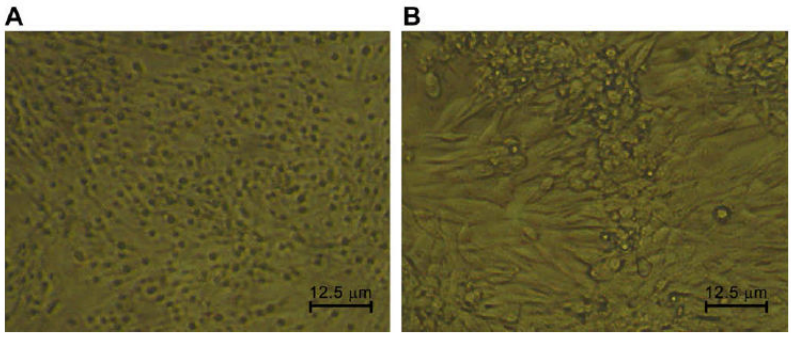


Fig. 4. Morphological differences in Chinese hamster ovary (CHO) cells in culture photographed by phase contrast microscopy. (A) Control cells show aberrant morphology induced by serum deprivation. (B) After serum deprivation, cells treated with 50 μg/ml of *Schistosoma haematobium* total antigen (Sh), displayed polarised lamellipodia. The black bars represent 12.5 μm.

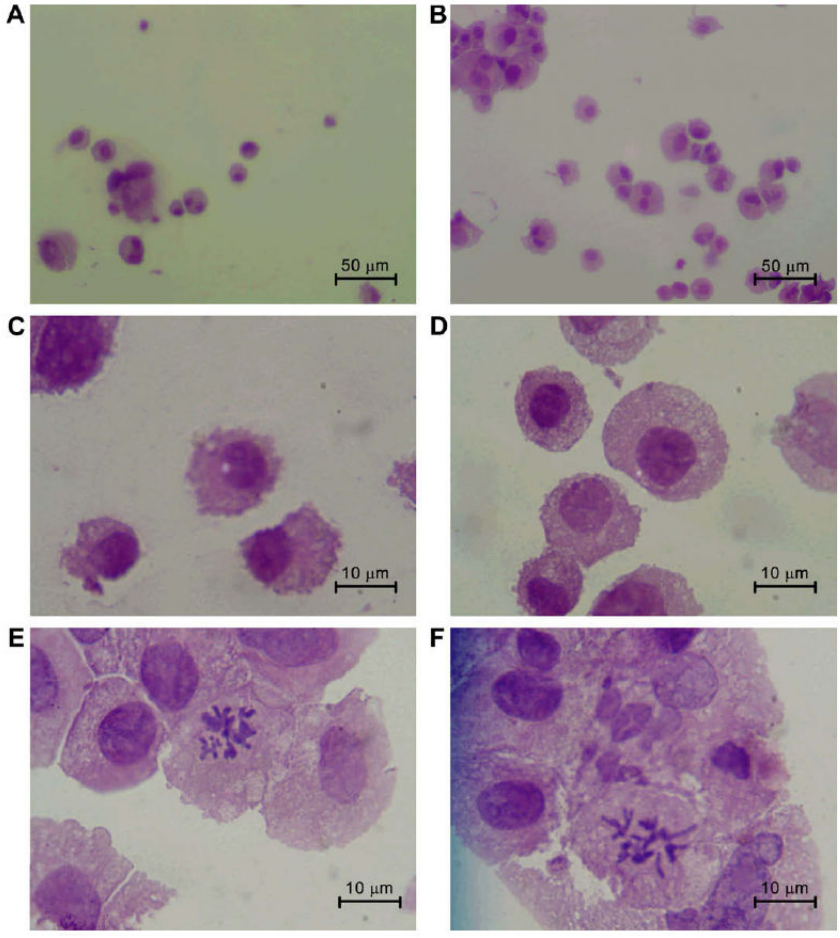


Fig. 5. Morphological differences in Chinese hamster ovary (CHO) cells photographed after cytospin followed by H & E staining. (A) Control cells (200×). (B) Cells treated with *Schistosoma haematobium* total antigen (Sh) are bigger, with larger nuclei and cytoplasms (200×). The black bars represent 50 μm. (C) Control cells (1000×). (D) The cell membranes of treated cells presented more integrity than control cells (1000×). (E) and (F) Sh-treated cells showing mitotic figures. The black bars represent 10 μm.

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4. Discussion

We have previously demonstrated that Sh has the potential to induce tumour development in a xenograft model, in which Sh-treated CHO cells formed tumours with similar phenotypes in all inoculated nude mice (Botelho et al., 2009). Infection with this parasite is largely associated with SCC of the bladder (Salim et al., 2008). Nevertheless, the mechanisms that cause cancerization of the bladder urothelium by *S. haematobium* are largely unexplained. In the present study we characterised the effect of Sh in mammalian cells using biological cell approaches normally used in carcinogenesis studies such as proliferation, apoptosis, cell cycle alterations, migration and invasion.

First we used Sh in cell cultures and showed that Sh increases proliferation. We have no references to date in the literature to compare our results concerning schistosomes and epithelial cell proliferation. However, the results of Thuwajit et al. (2004) reported that excretion/secretion (ES) products from *Opistorchis viverrini*, a liver fluke associated with cholangiocarcinoma, induced cellular proliferation in mouse fibroblast NIH-3T3 cells. Kim et al. (2008) used the human epithelial cell line HEK293 to examine the in vitro effect of ES products of *Clonorchis sinensis*, another liver fluke also associated with cholangiocarcinoma, on the growth of bile duct epithelial cells. The cells proliferated in the presence of ES products in a dose-dependent manner (Kim et al., 2008). Mouse fibroblasts co-cultured with *O. viverrini* adult worms underwent more than fourfold greater proliferation than cells in media alone. Moreover, human cholangiocarcinoma cell lines underwent excessive proliferation upon stimulation with *O. viverrini* soluble ES products. Regarding schistosomes, Wyler and Tracy (1982) indicated that total antigen from *Schistosoma* eggs contains a biologically active molecule(s) capable of directly stimulating fibroblasts in vitro. Schistosomal granulomas caused by entrapment of the parasite eggs in the bladder are also a rich source of cytokines and growth factors, whose roles in connective tissue proliferation and activation as well as in fibroplasias and SCC of the bladder

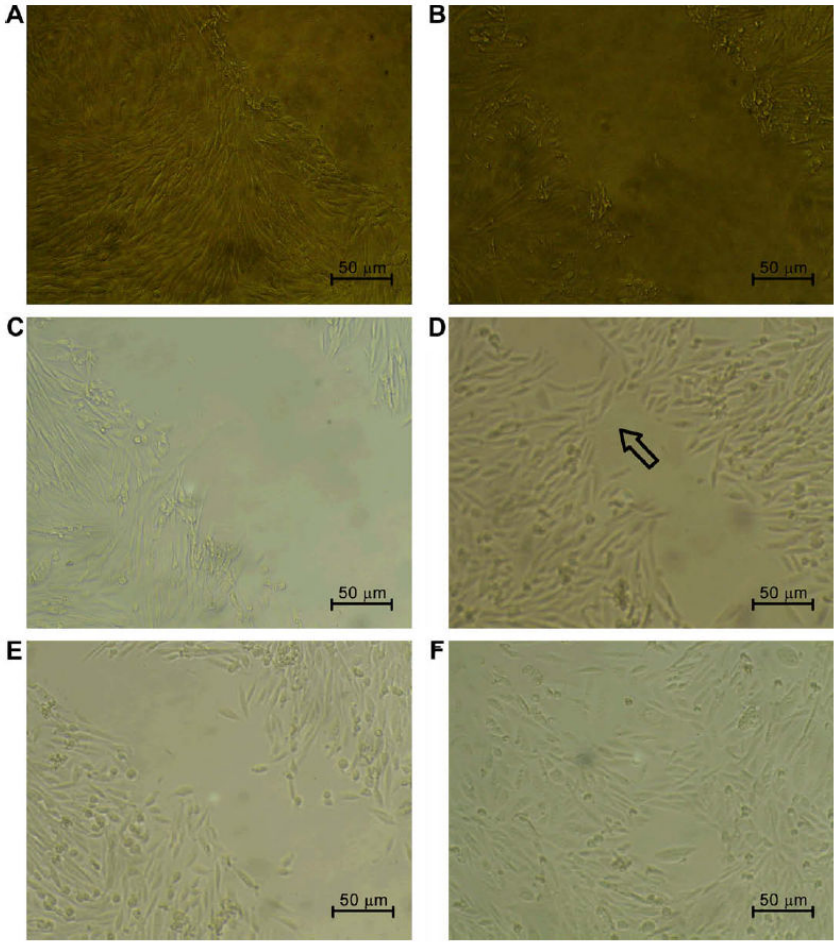


Fig. 6. Cell migration observed by wound closure assay of Chinese hamster ovary (CHO) cells. (A) Control and (B) treated with 50 µg/ml of *Schistosoma haematobium* total antigen (Sh) at the beginning of the experiment. (C) Control cells after 24 h. (D) Sh-treated cells begin bridging (arrow) to close the wounded area after 24 h. (E) Control cells had not yet closed the wound after 48 h. (F) Sh-treated cells completely closed the wound after 48 h. The black bars represent 50 µm.

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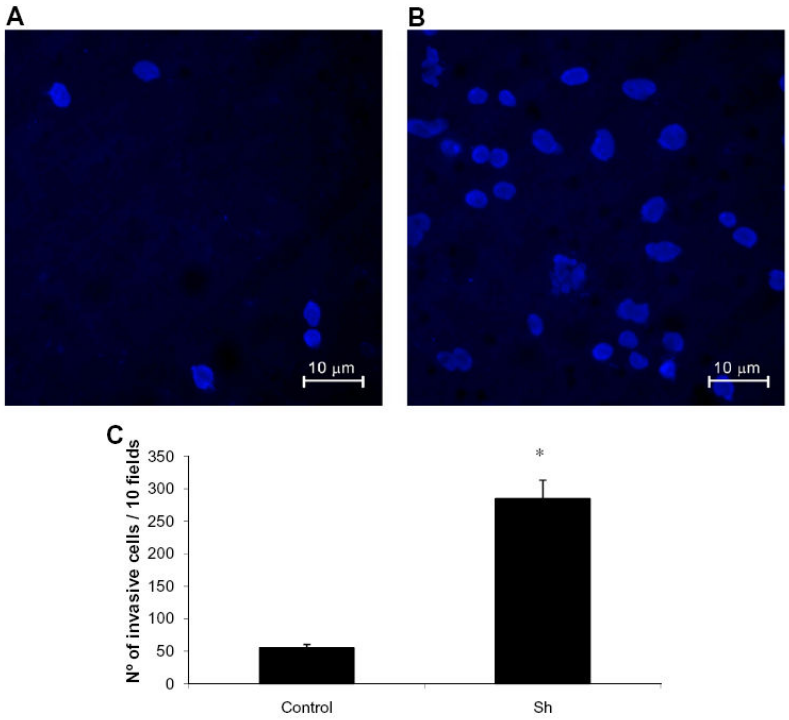


Fig. 7. Effect of *Schistosoma haematobium* total antigen (Sh) on cell invasion. (A) Microphotographs of invasive cells of controls and (B) Sh-treated cells. Both panels have the same magnification. Note the increased number of cells stained by DAPI in the Sh-treated cells. The white bars represent 10 µm. (C) Sh induced the invasion of treated cells fivefold more than control cells ($P < 0.01$; control versus Sh-treated cells).

are well established. Extensive proliferation of inflammatory cells of myeloid origin associated with schistosomal granulomas is also well known (Chiarini et al., 2006). In addition it has been shown that prolonged stimulation of excessive proliferation of urinary bladder epithelial cells in rats results in carcinoma formation (Otori et al., 1997). We have shown increased *S*-phase and *G2/M*-phase of the cells treated with Sh. We have also shown loss of p27 as a direct result of cell treatment with Sh. Defective regulation at the p27 checkpoint in the cycle can result in uncontrolled cellular proliferation. This is in agreement with the report of Kim and collaborators (2008) who demonstrated that HEK 293 cells treated with ES products of *C. sinensis* increased *G2/M*-phase, indicating that the G1 to S and S to G2/M transitions had occurred.

Apoptotic cell loss in carcinogenesis has been examined using the TUNEL method (Takaba et al., 2000). We used this method to analyse apoptosis in the present work and found it dramatically decreased in cells after treatment with Sh. Several pathogens, including schistosomes, have also been shown to manipulate apoptosis to control host cell death (Rutitzky et al., 2003). Alterations in the balance between cell proliferation and apoptosis may reflect an important mechanism of carcinogenesis (Otori et al., 1997). In accordance, apoptosis is rarely observed in either *O. viverrini*-infected hamster livers or in biliary cells that are co-cultured with flukes in vitro. Biliary cell damage by *O. viverrini* likely stems from the actions of oxygen radicals such as nitric oxide (NO). NO not only induces DNA damage but has been reported to mediate DNA repair inhibition. Moreover, NO has also been demonstrated to inhibit apoptosis. All of these manifestations facilitate carcinogenesis. Anti-apoptosis has been described in infection-associated cancers such as those caused by *Helicobacter pylori*. In the case of *O. viverini* infection, DNA damage is caused in biliary epithelial cells while apoptotic mechanisms are deregulated, resulting in genetic alterations which may become fixed, leading to malignant transformation (Sripa et al., 2007). *Schistosoma haematobium* infection is likely to cause bladder cancer by the same mechanism. Salim et al. (2008) suggested a strong correlation between *S. haematobium* infection and increased levels of oxidative stress accompanied by continuous DNA damage and repair in urothelial carcinomas, all directly correlating with elevated NO. The *bcl-2* product plays a role as an inhibitor of apoptosis; it contributes to oncogenesis by suppressing signals that induce apoptotic cell death. In our work we have found increased expression of Bcl-2 in Sh-treated cells compared with controls. Swellam and collaborators (2004) found that Bcl-2 was significantly expressed in bladder tumours associated with schistosomiasis compared with other bladder tumours. These observations, besides confirming our results, detect a potential role for bcl-2 expression in schistosomal carcinogenesis (Swellam et al., 2004).

We also found alteration of cell morphology after treatment with Sh. In *O. viverrini*, the morphology of NIH-3T3 mouse fibroblasts was altered after incubation with ES products (Thuwajit et al., 2004). These researchers explained the phenomenon as an ability of the cells to proliferate in the limited culture area by acquiring a refractive and narrow shape. This is in agreement with the widely accepted concept that tumours evolve by cloning selection of cell populations that proliferate in an unconstrained manner and compete for nutrients or space (Anderson et al., 2006), forcing them to alter their morphology.

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Understanding the mechanisms that lead to the invasion of host tissues by a growing population of neoplastic cells is a major task of cancer research (Santra et al., 1997). In the present work we have used in vitro assays to evaluate migration and invasion of Sh-treated cells and found those increased compared with controls. As Oliveira and collaborators (2006) have stated, the Matrigel invasion assay used in this study is by no means the equivalent of invasion in the in vivo situation. These investigators have repeatedly emphasised that all elements in the ecosystem need to be taken into account when analysing the phenotypes of cells. Nevertheless, the Matrigel assay we used contains some of the elements of the in vivo ecosystem of invasion, such as collagen type IV, laminin and entactin. Furthermore, this assay covers some of the cellular activities implicated in invasion, such as three-dimensional migration through matrices and proteolysis (Oliveira et al., 2006). We have demonstrated that Sh induces invasion of CHO cells through the use of a Matrigel invasion assay. This could lead to extracellular matrix degradation and subsequent invasion of cancer cells, suggesting a role for Sh in later stages of bladder carcinogenesis.

Taken together, the effects observed in Sh-treated cells seem to be interconnected. In the present work we used total antigen of *S. haematobium* because we previously reported a marked response by CHO cells to this extract (Botelho et al., 2009). There is no report in the literature that associates schistosome adult worms to cancer. It is intriguing that the worm extracts caused the alterations described above in normal epithelial cells similar to the ones described in cancer cells. Nevertheless, schistosome worms and egg antigens share common molecules (Soliman et al., 2003; Faria-Pinto et al., 2008). Although we do not yet fully understand the physiological functions of Sh or the molecular disease mechanism in *Shistosoma*-associated bladder cancer, the present study revealed intriguing novel aspects. Through its effects in cell biology, Sh is likely to participate in a number of carcinogenesis-mediated processes, such as increased cell proliferation, loss of p27, decreased apoptosis, increased expression of Bcl-2, increased migration and invasion, all of which are processes needed for cancer cell survival. The effects of Sh on the cell cycle may contribute to the high proliferation rate and accumulation of genetic changes during schistosomiasis-associated tumourigenesis. Loss of p27 may be the reason for the uncontrolled proliferation seen in Sh-treated cells and could be involved in bladder cancer-associated schistosomiasis.

Given the strong association between this parasite and bladder cancer, identification of the *S. haematobium* carcinogenic molecules and the molecular pathways they trigger would prove informative.

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PAPER III

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ORIGINAL ARTICLE

Tumourigenic effect of *Schistosoma haematobium* total antigen in mammalian cells

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Summary

Schistosoma haematobium is endemic in several regions of Africa and has been shown to be associated with predominantly squamous cell bladder carcinoma. The mechanisms underlying the association between *S. haematobium* and bladder squamous cell carcinoma is largely unknown. All the reports so far, demonstrate exclusively an epidemiological evidence linking *S. haematobium* infection with squamous cell bladder carcinoma. We hypothesized that these parasite antigens might induce tumourigenesis. For this, we used normal mammalian cells of Chinese hamster ovary (CHO) and treated the cells in culture with *S. haematobium* total antigen (Sh). Our results showed increased proliferation in Sh-treated cells in comparison with the controls. The CHO cells exposed to Sh were inoculated subcutaneously into male nude mice and formed sarcomas ($n = 5/5$). The cells from the sarcomas expressed vimentin filaments and were negative to cytokeratin. Our results demonstrate for the first time that *S. haematobium* antigens induce tumour development in nude mice.

Keywords
Schistosoma haematobium, Chinese hamster ovary cell line, cell proliferation, nude mice, argyrophilic nucleolar organizing region

Human urothelial carcinomas constitute the vast majority of bladder cancers in most of the world. Smoking, exposure to certain dyestuffs, aromatic amines, rubber production, painting and leatherwork seem to be associated with an increase risk of bladder cancer in developed countries (Kogevinas & Trichopoulos 2002). *Schistosoma haematobium* is endemic in several regions of Africa and has been shown to be associated with predominantly squamous cell

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bladder carcinoma, a rare subtype in the western world (Gutiérrez *et al.* 2004). The epidemiological association between *S. haematobium* and squamous cell carcinoma is based both on case control studies and on the close correlation of bladder cancer incidence with prevalence of *S. haematobium* infection within different geographical areas. The mechanisms in which urinary schistosomiasis induces bladder cancer are incompletely understood. Both chemical and physical carcinogenic pathways have been implicated and it has been suggested that chronic mechanical irritation by calcified eggs deposited in the bladder epithelium invokes changes in the urinary tract epithelium (Hodder *et al.* 2000). Chinese hamster ovary cells (CHO cells) are a cell line derived from Chinese hamster ovary cells often used in biological and medical research. We hypothesized that the parasite total antigen might induce tumourigenesis. In this study, we employed a cell biology approach to investigate the involvement of *S. haematobium* total antigen in tumourigenesis. In this paper, we document evidence for the oncogenic potential of *S. haematobium* total antigen by its treatment of CHO cells resulting in increased proliferation and tumourigenesis in nude mice. To characterize the tumours developed in mice, we made a histological study and examined argyrophilic nucleolar organizing region (AgNOR), cytokeratin and vimentin expression. To our knowledge, this is the first report demonstrating alterations of normal mammalian cells and tumourigenesis as a direct effect of *S. haematobium* antigens. Strategies to block these antigens may contribute to halt the growth of squamous cell bladder carcinomas associated with *S. haematobium*.

Methods

Schistosoma haematobium total antigen production

Schistosoma haematobium adult worms were collected by perfusion of the hepatic portal system of golden hamsters at 7 weeks after infection with 100 cercariae. The worms were suspended in phosphate-buffered saline (PBS) and then sonicated. The protein extract was then ultracentrifuged and the protein concentration was estimated using a micro BCA (bicinchoninic acid) protein assay reagent kit.

Cell culture

Chinese hamster ovary (CHO) cell line was obtained from IPATIMUP. Chinese hamster ovary cells were cultured and maintained at 37 °C in a 5% CO2 humidified atmosphere in CHO medium (Sigma) with 10% FBS (Fetal Bovine Serum) and 1% penicillin/streptomycin (Sigma). Cells were passaged every 5 days. Before treatments, cells were serum-starved for 16 h. The cells were treated with Sh for 24 h. Control cells were treated with serum free medium. After treatment, the cells were harvested by trypsinization and counted under microscope after trypan blue staining.

Proliferation assay

The CellTiter 96 AQ non-radioactive cell-proliferation assay (Promega, Madison, WI, USA) was used to assess cell proliferation. The assay is composed of the tetrazolium compound MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and an electron coupling reagent, PES (phenazine ethosulfate), to produce a colorimetric change. MTS is reduced by viable cells to formazan, which can be measured with a spectrophotometer by the amount of 490 nm absorbance. Formazan production is time dependent and proportional to the number of viable cells. Chinese hamster ovary cells were cultured in 0.1 ml CHO media in 96-well flat-bottomed plates. Cultures were seeded at 1×10^4 cells/well and allowed to attach overnight. After the indicated time of incubation with the appropriate medium, 20 μl reagent was added per well, and cells were incubated 1 h before measuring absorbance at 490 nm. Background absorbance from the control wells was subtracted. Studies were performed in triplicate for each experimental condition.

Nude mice

The experiments were carried out 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). Male N:NIH(s)II-nu/nu mice of 4–6 weeks old were obtained from IPATIMUP. These animals were maintained under sterile conditions throughout the experiment (temperature 24 ± 2 °C, relative humidity 55 ± 5% and a 12 h photoperiod) in polycarbonate cages. They were fed sterilized autoclave rodent feed and water *ad libitum*.

Tumourigenesis

Eight mice were randomly divided into two experimental groups, group 1 with five animals and group 2 with three animals. Chinese hamster ovary cells were suspended in 200 μl of PBS and inoculated subcutaneous in the neck into the mice from group 1. Animals from group 2 were used as

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the control group; these animals were injected with CHO cells not treated with Sh.

After transplantation, the size of each tumour mass developed was measured with a calliper twice a week and tumour volumes were calculated as previously described (Tang *et al.* 2003). On day 22 after cell administration, all animals were killed under anaesthesia by intracardiac injection of pentobarbital (40 mg/kg) and tumours were removed from each animal. The tumours were examined macroscopically, cut in sections with 4 mm, fixed in 10% formalin and then embedded in paraffin. Haematoxylin and eosin (H&E) stained sections were prepared and examined to classify the neoplasic transformation.

Immunohistochemical procedure and evaluation

Immunohistochemistry was carried out with the streptavidin–biotin-peroxidase complex technique as previously described (Oliveira *et al.* 2008) using Ultravision detection system anti-polyvalent, HRP (LabVision Corporation, Fremont, CA, USA) for pan Cytokeratin, Cytokeratin (CK) 7, CK 20, Anti-Cytokeratin (CAM 5.2) and vimentin, using specific primary antibodies raised against pan-cytokeratin (cocktail of cytokeratins 5, 6, 8 and 18, clones 5D3 and LP53; Abcam Inc, Cambridge, MA, USA) diluted 1:100, CK 7 (clone OV-TL 12/30; Neomarkers, Fremont, California, USA) diluted 1:100, CK 20 (clone Ks20.8; Neomarkers) diluted 1:20, CAM 5.2 (clone CAM 5.2; Becton Dickinson, Franklin Lakes, NJ, USA) diluted 1:5 and vimentin (clone V9; Neomarkers) diluted 1:100. Briefly, deparaffinized and rehydrated sections were immersed in PBS. Slides were then incubated with 3% hydrogen peroxide in methanol for 10 min. Slides were incubated in Ultravision block solution (Neomarkers) for 10 min at room temperature before overnight incubation with the primary antibody. Then, sections were sequentially washed in 1× PBS with 0.02% Tween-20 incubated with biotinylated goat anti-polyvalent antibody for 10 min, streptavidin peroxidase for 10 min, and developed with 3,3-diaminobenzidine for 10 min. Slides were counterstained with Mayer's haematoxylin (Merck, Dermstadt, Germany) and mounted with entelan (Merck). Negative controls were carried out by omitting the primary antibody.

Cytokeratins and vimentin expression were evaluated as positive or negative, independent of marker extension.

Argyrophilic nucleolar organizing region histochemical procedure and evaluation

Argyrophilic nucleolar organizing region (AgNOR) histochemical staining was performed using a previously described modified 1-step silver-staining technique (Ploton *et al.* 1986). In brief, 2-μm sections of formalin-fixed paraffin-embedded tissue were cut, deparaffinized in xylene, rehydrated in graded ethanol and rinsed in distilled water. Slides were incubated for 30 min at room temperature in the dark with freshly made AgNOR staining solution consisting of 0.02-g gelatin in 1 ml of 1% formic acid and 1-g silver nitrate in 2 ml of distilled water. Following AgNOR staining, slides were rinsed with distilled water, dehydrated with graded ethanol and xylene and coverslipped.

To determine the average AgNOR count/cell in each tumour, AgNORs were counted in 1000 randomly selected neoplastic cells throughout the tumour at 1000× magnification. Individual AgNORs were resolved by focusing up and down while counting within individual nuclei. Average AgNOR counts/cells were then determined on the basis of averaging the counts within these 1000 random neoplastic cells as has been shown by numerous publications (Martin *et al.* 1992).

Statistical analysis

All statistical tests were performed using the SPSS 12 statistical software (SPSS Inc. USA). The results are presented as mean ± standard deviation. The values of AgNOR for various groups were compared using Student's *t*-test.

Results

Schistosoma haematobium *total antigen increased the proliferation of CHO cells* in vitro

To begin investigating the effect of *Schistosoma haematobium* total antigen (Sh) on cell viability and proliferation, CHO cells were seeded on 96 well plates, starved overnight, treated with increasing concentrations of Sh for 24 h, cultivated for 24, 48 and 72 h and then analysed by MTS assay (Figure 1). The growth curve shows that treated cells proliferated significantly faster and more than the control cells. These results suggest that the increased proliferation in CHO cells is a consequence of Sh treatment. We used the concentration of 50 μg/ml of Sh for the subsequent assays.

Animal general condition

No deaths attributable to the treatment were observed. All mice from group 1 had positive tumour growth 15 days after inoculation and were necropsied 22 days later. There was no tumour growth in control group. Sh allows the vigorous growth of CHO xenografts in nude mice (Figure 2).

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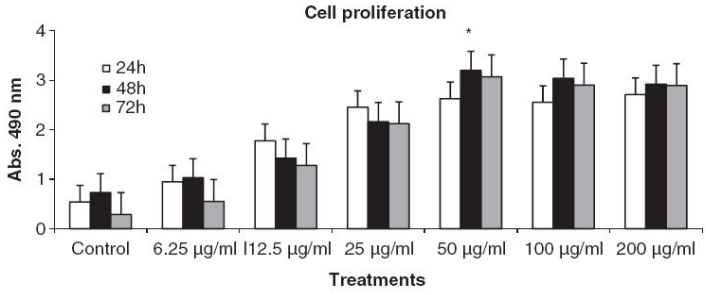


Figure 1 Cell proliferation assay of *Schistosoma haematobium* total antigen treated cells. The experiments were carried out in triplicate. The growth curve shows that treated cells proliferated significantly faster and more than the control cells. ($P < 0.01$; control *vs.* 50 µg/ml).

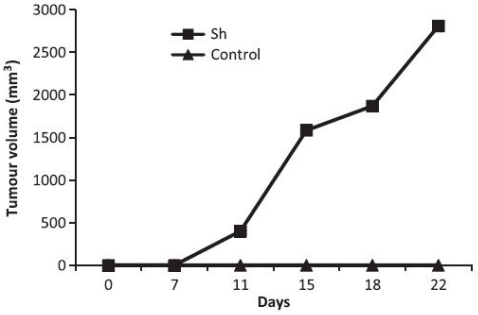


Figure 2 Analysis of the tumour volume induced by control Chinese hamster ovary (CHO) and *Schistosoma haematobium* total antigen (Sh) treated CHO in nude mice. Tumour volumes were measured at the indicated days after injection. Each point represents the mean of five individual determinations.

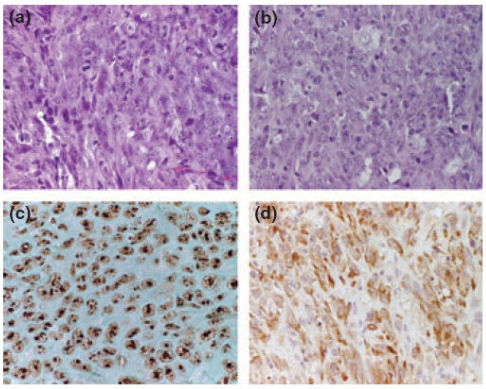


Figure 3 Histological findings of the tumour mass produced by inoculation of Chinese hamster ovary (CHO) cells exposed to *Schistosoma haematobium* antigen (a, b) mice sarcomas stained by Haematoxylin and eosin (H&E) (600×), (c) mice sarcoma stained by argyrophilic nucleolar organizing region (AgNOR) technique (600×) and (d) Immunohistochemistry of sarcoma showing vimentin filaments (600×).

Macroscopic and microscopic evaluation

All mice injected with Sh developed a solid, large multilobulated mass in interscapullary region. These tumours grew very fast and ulcerated overlying the skin. No macroscopic changes were observed in the interscapular region in the control group. The cut surface of the tumours was white and firm. Microscopic observation of these tumours showed a pleomorphic and fusocellular neoplasia with intensive inflammatory infiltrate, predominantly lymphocytes and neutrophils. We have also identified round cells with prominent nucleus, and exuberant nucleolus, with a histiocytic pattern and frequent mitosis. Necrotic foci were seen in these tumours. All tumours corresponded to sarcomas. There were no tumour metastases. Histological findings from mice inoculated with Sh can be observed in Figure 3.

Immunohistochemistry and histochemistry

Immunohistochemistry was performed on all tumours from group 1. These tumours strongly expressed vimentin filaments.

Table 1 Results of immunohistochemistry and histochemistry

		Immunohistochemistry		Histochemistry
Group	Tumour	Cytokeratins	Vimentin	AgNORs
1(Sh)	Sarcoma	–	+	1.97 ± 1.09*
1(Sh)	Sarcoma	–	+	1.94 ± 1.06
1(Sh)	Sarcoma	–	+	1.72 ± 0.9
1(Sh)	Sarcoma	–	+	1.88 ± 1.09
1(Sh)	Sarcoma	–	+	1.86 ± 1.12
2(control)	–	–	–	–
2(control)	–	–	–	–
2(control)	–	–	–	–

*Values are mean values ± SEM ($P > 0.05$, *t*-test); + positive, – negative.
Sh, *Schistosoma haematobium* total antigen; AgNORs, argyrophilic nucleolar organizing regions.

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The tumours were negative for the tested cytokeratins (Table 1 and Figure 3). Argyrophilic nucleolar organizing regions were visible as black or brown dots, with various sizes, within the nuclei of cells (Figure 3). The mean values of AgNORs were similar and not statistically different between tumours, these values are shown in Table 1.

Discussion

Here, we have used a xenograft model to show that Sh has the potential to induce tumour development. First we used Sh in cell cultures and showed that Sh increases proliferation. We have no references so far in the literature to compare our results. However, the results of Wyler and Tracy (1982) clearly indicate that total antigen from *Schistosoma* eggs contains a biologically active molecule(s) capable of directly stimulating fibroblasts *in vitro*. It has also been shown that prolonged stimulation of excessive proliferation of urinary bladder epithelial cells in rats results in carcinoma formation (Otori *et al.* 1997). Schistosomal granulomas are a rich source of cytokines and growth factors, whose role in connective tissue proliferation and activation as well as in fibroplasias is well established. Extensive proliferation of inflammatory cells of myeloid origin associated with schistosomal granulomas is also well known (Chiarini *et al.* 2006).

Chinese hamster ovary cells exposed to Sh described in this paper formed similar phenotypes in all inoculated nude mice. It is worth noting that all the formed phenotypes were sarcomas. The morphology of CHO cells is both epithelial-like and fibroblast-like. Our results suggest that Sh may induce the transformation of the fibroblast-like component of CHO cells into sarcomas. This result is not surprising as it has been shown that egg granulomas isolated from livers of mice infected with *Schistosoma mansoni* or *S. japonicum* and cultured *in vitro* released a fibroblast-stimulating substance which stimulated proliferation in resting dermal fibroblasts (Wyler & Tracy 1982). Schistosomal egg granulomas spontaneously secrete fibrogenic factors, suggesting that there exists a molecular link between granulomatous inflammation and hepatic fibrosis in schistosomiasis (Prakash *et al.* 1990). Hepatic fibrosis complicates the chronic granulomatous inflammatory reaction to *S. mansoni* eggs and is the major cause of morbidity and mortality in human schistosomiasis (Wyler 1983). This liver pathology is the terminal event of a complex pathophysiological cascade involving interactions between fibroblasts and both host and parasite products (Lammie *et al.* 1986) and is associated with prominent accumulations of fibroblasts (Dunn *et al.* 1986), reinforcing the present results.

Carcinogenesis is a complex process in which normal cell growth is modified as a result of the interaction of multiple factors. There is a strong evidence to suggest that biological carcinogens are a major cause of cancer in humans. It has been estimated that chronic infections by virus, bacteria and parasites contribute to 13% of world's cancer (Parkin *et al.* 1999).

Although helminths have been implicated in the aetiology of human cancer, the knowledge of the mechanisms by which parasites induce malignant transformation of the host cells is unclear. *Schistosoma haematobium* is considered to be carcinogenic to humans, with sufficient evidence for its role in causing carcinoma of the bladder (Parkin 2006). According to Herrera and Ostrosky-Wegman (2001), parasites might initiate carcinogenesis by direct action of genotoxic factors, either secreted by them or produced during the inflammatory response.

Inflammation is a common feature of helminthiasis in which inflammatory cells regenerate reactive oxygen species that, apart from killing invading pathogens, are capable of inducing genetic instability in normal surrounding tissue, which can lead to malignant transformation. As we show with AgNOR evaluation, the sarcomas developed in nude mice had a high proliferation index. Several authors reported that AgNOR staining is a simple and useful method for estimating tumour cell proliferation (Khan *et al.* 2006). Nucleolar organizer regions are focal aggregates of intranucleolar non-histone proteins that are associated with potential sites of ribosomal DNA transcription (Rüschoff *et al.* 1989). These proteins are easily localized by virtue of their argyrophilia. The mean number of AgNORs per nucleus accurately correlates with mitotic rate in tumour cell lines and AgNOR counts may thus provide indirect measurement of mitotic rate in tumours.

Further investigations using *S. haematobium* antigens may be useful to understand tumourigenesis associated with this parasite.

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UROLOGIC ONCOLOGY

Original article

Urothelial dysplasia and inflammation induced by *Schistosoma haematobium* total antigen instillation in mice normal urothelium

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Abstract

Objectives: Squamous cell carcinoma of the urinary bladder has been associated with *Schistosoma haematobium* infection in many parts of Africa. The epidemiologic association is based on case control studies and on the close correlation of urinary bladder cancer incidence with prevalence of *S. haematobium* infection within different geographic areas. A parasite-tumor linkage is further suggested by the predominance of squamous cell (as opposed to transitional cell) morphology of bladder carcinomas seen in *S. haematobium*-endemic areas. The cellular mechanisms linking *S. haematobium* infection with cancer formation are not yet defined. In the present study, we hypothesized that the parasite antigens might induce alterations in urothelium.

Materials and methods: We investigated the effects of *S. haematobium* total antigen in CD-1 mice normal bladders after intravesical administration of the parasite antigens. The bladders were analyzed histopathologically 20 and 40 weeks after treatment.

Results: Intravesical instillation of *S. haematobium* total antigens induces the development of urothelial dysplasia and inflammation.

Conclusions: In our work, we demonstrate for the first time that *S. haematobium* antigens are the direct cause of alterations in urothelium. © 2009 Published by Elsevier Inc.

Keywords: *Schistosoma haematobium*; Bladder cancer; Urothelial dysplasia; Inflammation; CD-1 mice

1. Introduction

Schistosomiasis is a common parasitic disease that ranks second only to malaria as a global cause of morbidity and mortality. It occurs in ~~74~~ tropical and subtropical countries and affects about 10% of the world's population. The prevalence of this infection remains high despite the availability of efficacious antischistosomal drugs. Individuals become infected with the parasite at an early age by exposure to the free-swimming larval stage (termed cercariae) in contaminated water [1]. The life cycle involves both an aquatic snail intermediate ~~(Biomphalaria spp.)~~ and a human definitive host. Mice and hamsters can be used to maintain the life cycle in the laboratory. Male and female adult worms are found in the venules draining the bladder. Eggs pass through the mucosa and out of the body urine. The eggs hatch in fresh water, and motile miracidia actively search for snails. Following penetration into the snail host, miracidia differentiate into sporocysts. Sporocysts proliferate asexually in the snail, eventually releasing motile clonal cercariae into the water. Cercariae penetrate the unbroken skin of a mammalian host, and then migrate through the bloodstream to the hepatic portal system where they develop into adults. In the laboratory, the entire life cycle takes 90 to 120 days to complete [2].

Schistosomiasis is the first in prevalence among all water-borne infectious diseases and one of the main occupa-

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tional risks encountered in the rural endemic areas of the world. Schistosomiasis-associated bladder cancer defines a characteristic pathologic and histogenic nature that differentiates it from bladder cancer of nonschistosomal origin. It shows an increased incidence of squamous cell carcinoma as opposed to transitional cell carcinoma, a lower mean age at diagnosis, and a higher male/female ratio. Moreover, in association with schistosomiasis, cancer may originate throughout the bladder but rarely in the trigone, a frequent site of tumor origin in nonschistosomal cases [3]. The tumors are often first seen in an advanced stage, arising from the posterior bladder wall and vault. The trigone is only affected in 8.5% of the cases [4]. The reason accounts for the striking deformities in the ureters, including medial deviation, a straight lumbar course, and in the pelvic segment a bowed appearance with medial and cranial displacement at the vesico-ureteric junction. Stasis and dilation of the upper urinary tract are present in the absence of mechanical obstruction and these changes in the upper urinary tract are due to functional derangement of the ureters [5]. Human bladder cancer is the fifth to the seventh most common cancer in Western countries. In many tropical and subtropical areas, however, it is the first among all types of cancer, mainly due to the endemic parasitism [3].

The involvement of *S. haematobium* infection in bladder cancer is strongly supported. Various hypotheses have been proposed to explain why *S. haematobium* infections predispose to bladder cancer. It has been suggested that environmental carcinogens, such as cigarette smoke or pesticides (many infected individuals are farmers) could play a role in the development of cancer by interacting synergistically with the infection. Another more favored suggestion implicates urinary nitrosamines as initiating agents. In support of this hypothesis are data showing that N-nitroso compounds are elevated in the urine of infected individuals to levels greater than those observed in uninfected individuals. These N-nitroso compounds may come from exogenous sources (e.g., diet) or, alternatively, be formed endogenously through the action of bacteria or stimulated inflammatory cells, both commonly present in the bladder of these patients. Another factor that may play a major role in bladder carcinogenesis in schistosomiasis patients is the presence of continuous physical irritation and inflammation produced by schistosomal eggs in the bladder mucosa [1,6–8].

We have previously demonstrated that normal cells treated in the laboratory with *S. haematobium* total antigen acquire cancer-like characteristics. In fact, these cells presented rapid, uncontrolled division, high resistance to death, and an abnormal capability to migrate [9] and, when injected into mice with no immune system, led to the formation of tumors. Crucially, animals injected with nonexposed cells showed no growth [10].

In the present study, we hypothesized that the parasite total antigen might present carcinogenic potential. Therefore, we employed an in vivo strategy to investigate the involvement of *S. haematobium* total antigen in carcinogenesis of the bladder of CD-1 mice. In this paper, we document evidence for the first time of the carcinogenic effect of *S. haematobium* total antigen by its instillation in the bladders of CD-1 mice, inducing the development of dysplasia in these animals.

2. Materials and methods

2.1. Animals

Eight-week-old female golden hamsters (LVG/SYR) and CD-1 mice were provided by Charles River (Barcelona, Spain). Animals spent 1 week being acclimated under routine laboratory conditions before starting the experiments. They did not receive any treatment prior to the study. Hamsters were kept in separate cages, and mice were kept in 6 litter mates to a cage. 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).

2.2. Experimental infections

Urine was collected from *S. haematobium*-infected individuals. The individuals were living in Angola, an endemic area for schistosomiasis. Following instruction in midstream urine collection, urine samples were then collected from each individual. *S. haematobium* infection was detected by microscopic observation of the eggs in the sediment of centrifuged urine. Informed consent from patients was obtained. The eggs were hatched and with the resulting miracidia, snails from susceptible species were infected.

Golden hamsters were experimentally infected with 100 cercariae by tail immersion. The control animals consisted of littermates. The cercariae were obtained by shedding of snails infected with miracidia.

2.3. S. haematobium total antigen (Sh) production

Angolan strain of *S. haematobium* adult worms were collected by perfusion of the hepatic portal system of golden hamsters at 7 weeks after infection with 100 cercariae. The worms were suspended in PBS and then sonicated. The protein extract was then ultracentrifuged and the protein concentration was estimated using a micro BCA protein assay reagent kit.

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2.4. Experimental design

Forty mice were randomly divided into 6 experimental groups. Groups 1 and 3 ($n = 10$) were Sh-instilled animals. Groups 2 ($n = 5$) and 4 ($n = 5$) were control animals with their bladders instilled with saline only. Groups 1 and 2 were sacrificed 19 weeks after treatment; group 3 and 4 were sacrificed 32 weeks after treatment. For the duration of the study, the mice's state of health was monitored daily.

For intravesical instillation of the parasite total antigen, 50 μg/ml of Sh was introduced directly into the urinary bladders of female mice via the urethra according to the procedure previously described [11]. Briefly, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). The bladder was catheterized via the urethra with a 24 gauge plastic intravenous cannula and the bladder mucosa was then traumatized to remove the umbrella cells of the urothelium by instilling 0.1 ml of 0.1N HCl solution for 15 s, neutralized with 0.1 ml of 0.1N KOH, and flushed with sterile saline.

A total of 150 μl of Sh with a concentration of 50 μg/ml was instilled via the cannula. The dwell time after instillation was approximately 1 hour. The mice were turned 90° every 15 minutes to facilitate whole bladder exposure to the Sh intravesically instilled. One hour after the instillation, micturition of the mice was induced by light abdominal massage so that the duration of exposure was constant. Body temperature was maintained with a homoeothermic bandage. All the experiments described herein were carried out under aseptic conditions and performed once during this study.

All animals were sacrificed by intraperitoneal administration of sodium pentobarbital (130 mg/Kg). Complete necropsies were carefully conducted. All organs were examined macroscopically for any changes. The urinary bladders were inflated in situ by injection of 10% phosphate buffered formalin (150 μl), ligated around the neck to maintain proper distension, and then were immersed in the same solution for 12 hours. After fixation, the formalin was removed, the urinary bladder was cut into two strips, and routinely processed. Tissue sections (2 μm) from urinary bladder, spleen, liver, and lung were stained with haematoxylin and eosin (HE) to characterize changes and inflammatory infiltrate.

2.5. Analysis and statistics

A descriptive study was performed for all the variables included in the study. Statistical analysis was performed using the SPSS 12.0 statistical package for Windows (SPSS Inc., Chicago, IL). The differences in incidence of lesions between groups were assessed by use of Fisher's exact probability test or the χ^2 test. A $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. Animals' general aspect

During the experimental work, no animal died, and all animals exhibited normal cage activity. The weight of the animals remained normal throughout the experiment (data not shown).

3.2. Macroscopic and microscopic evaluation

The bladders from all groups showed no gross lesions. No macroscopic changes were seen in the liver, lungs, or kidneys of all animals. The mean liver, kidney, and bladder weights, as well as relative liver, kidney, and bladder weights at the end of the study did not show differences (data not shown).

The bladder histopathologic findings observed in all groups are given in Table 1 and Figs. 1 and 2. Bladder histopathology T1,F1-2 of animals instilled with Sh was characterized by extensive diffuse or nodular infiltrate composed predominantly of lymphocytes admixed with variable number of eosinophils, and by absence of umbrella cells, nodular hyperplasia, and dysplasia. The nodular hyperplasia exhibited budlike proliferated urothelial cells extending into the lamina propria. After 20 weeks of treatment, 30% of these animals displayed urothelial dysplasia, a low grade intra-urothelial neoplasia) (LGIUN), whereas after 40 weeks of treatment we found 70% of the bladders with this LGIUN, a noninvasive malignant flat lesion in the urothelium of these animals. Mastocytes were only found in the bladders of animals sacrificed after 40 weeks. No bladder histopathologic changes were observed in animals from control groups.

Table 1
Histopathologic findings in the Sh instilled bladders and controls

Group	n	W	N	U	I	L	M	D
Group 1 (Sh treated)	10	20	7/10 (70%)	5/10 (50%)	9/10 (90%)	9/10 (90%)	0/10 (0%)	3/10 (30%)
Group 2 (control)	10	20	10/10 (100%)	9/10 (90%)	2/10 (20%)	2/10 (20%)	0/10 (0%)	0/10 (0%)
Group 3 (Sh treated)	10	40	0/10 (0%)	0/10 (0%)	10/10 (100%)	9/10 (90%)	4/10 (40%)	7/10[a] (70%)
Group 4 (control)	10	40	10/10 (100%)	10/10 (100%)	3/10 (30%)	3/10 (30%)	0/10 (0%)	0/10 (0%)

n = number of animals; W = weeks between treatment and sacrifice; N = normal urothelium; U = presence of umbrella cells; I = inflammatory infiltrate; L = infiltration of lymphocyte; M = infiltration of mastocytes; D = dysplasia.
[a] $P = 0.001$, group 3 vs. group 4.

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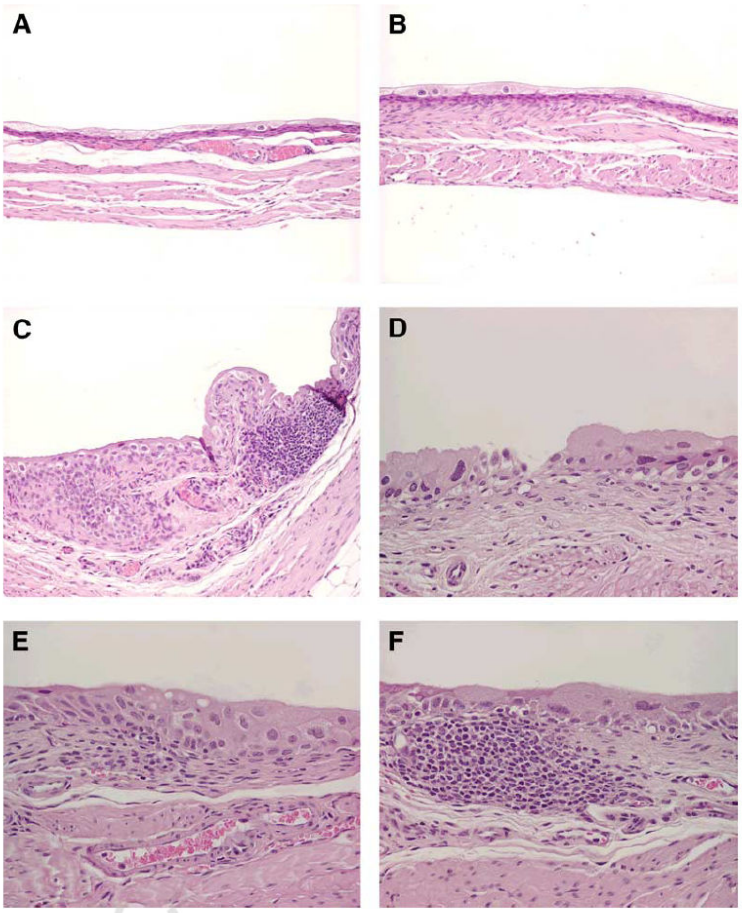


Fig. 1. Histopathologic findings in animals after 19 weeks of treatment. (A) and (B) Control (200×); (C) urothelium displaying an inflammatory nodule and a lesion with characteristics of nodular hyperplasia (200×); (D) denudation and dysplasia (400×); (E) inflammatory infiltrate and dysplasia (400×); (F) inflammatory nodule and dysplasia (400×).

No microscopic changes were seen in the spleen, lung, kidney, and liver in samples obtained from all animals.

4. Discussion

Here, we have used a CD-1 mice model to show that *Schistosoma haematobium* total antigen has a carcinogenic ability. Intravesically administration of Sh gave rise to a high incidence of urothelial dysplasia. The urothelium of the animals exposed to Sh described in this paper presented similar phenotypes. The urothelium of these animals presented dysplasia, a noninvasive malignant flat lesion, in 70% of the tested subjects after 40 weeks of treatment. Our results suggest that Sh induce the malignization of the urothelium.

Carcinogenesis is a complex process in which normal cell growth is modified as a result of the interaction of multiple factors. There is a strong evidence to suggest that biological carcinogens are a major cause of cancer in humans. It has been estimated that chronic infections by virus, bacteria, and parasites contribute to 13% of the world's cancer [12]. The mech-

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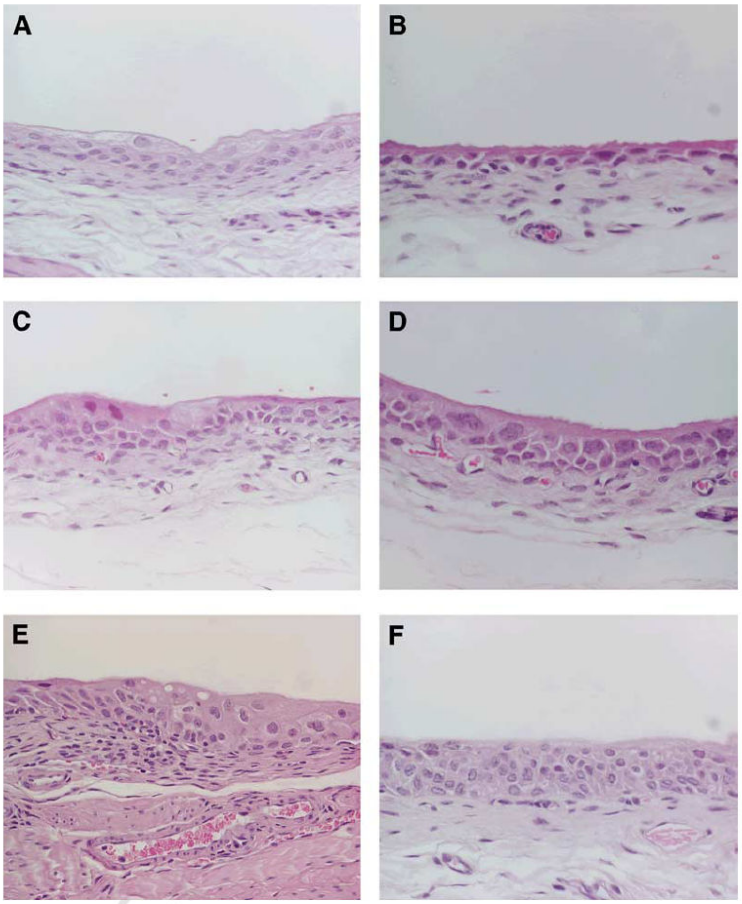


Fig. 2. Histopathologic findings in animals after 32 weeks of treatment. (A) control (400×); (B) dysplasia (600×); (C) dysplasia (400×); (D) urothelium displaying a lesion with characteristics of reactive atypia (600×); (E) inflammatory infiltrate and dysplasia (400×); (F) urothelium displaying a lesion with characteristics of simple hyperplasia with reactive atypia (600×).

anisms of carcinogenesis associated with infection and inflammation have not been fully elucidated [13].

Although helminths have been implicated in the etiology of human cancer, the knowledge of the mechanisms by which parasites induce malignant transformation of the host cells is unclear. *Schistosoma haematobium* is considered to be carcinogenic to humans, with sufficient evidence for its role in causing carcinoma of the bladder [12,14]. According to Herrera and Ostrosky-Wegman [15], parasites might initiate carcinogenesis by direct action of genotoxic factors, either secreted by them or produced during the inflammatory response. Activation of inflammatory cells may cooperate to induce cancer in many cases of infection- and inflammation-associated cancers [13].

It is generally recognized that the pathogenesis of malignancy involves 3 distinct and sequential effects on the target cells; initiation, promotion, and progression. The first stage is thought to involve the formation of damage in the DNA resulting in a somatic cell mutation. The essential feature of promotion, however, is the induction of proliferation in the initiated cells by agents that are not necessarily carcinogenic per se [3]. The sequence of lesions identified via histopathology between initiation and promotion are designated as preneoplastic lesions and/or benign neoplasias

[16]. Their transformation into malignant lesions is the last of the stages of carcinogenesis and is the most extended—it is labeled progression [17,18]. Several workers have proposed multistep and multifactorial models for bladder cancer development in schistosomiasis patients. These models attribute the initiation of carcinogenesis to low doses of nitrosamines or some other environmental carcinogen, with the infection supplying the proliferative stimulus to drive the expansion of clones of initiated cells. Although animal studies with this parasite infection have been difficult to perform, data obtained in a study by Hicks et al. [19] support such a model. Their data showed that, although proliferative and inflammatory changes occurred in infected animals without carcinogen exposure, the nitrosamine treatment was necessary before early neoplastic changes (carcinoma in situ) became apparent. The carcinogen treatment alone was too low to produce changes in the tissue. Also left unresolved is the mechanism by which the infection was contributing to the carcinogenic process. It is not known whether the inflammation itself was important or weather the infection was working through its effect on proliferation [1]. The questions raised by these researchers are now answered. We demonstrate for the first time that the carcinogenic process in the bladders of the animals exposed to parasite antigens is independent of the effect of nitrosamines. In our work, it is patent that the components of the parasite induce inflammation. Inflammation is a common feature of helminthiasis in which inflammatory cells regenerate reactive oxygen species (ROS) and reactive nitrogen species (RNS) which, apart from killing invading pathogens, are capable of inducing genetic instability in normal surrounding tissue that can lead to malignant transformation [13]. At low concentrations, these ROS act as intra- and intercellular second messengers modulating cellular functions, including proliferation, apoptosis and gene expression [20]. It is known that inflammatory response due to infectious diseases cause inducible nitric oxide synthase (iNOS-mediated oxidative stress during carcinogenesis [13], and that the mechanism for tumor development in the urinary bladder of *Schistosoma haematobium* infected patients could be explained by the endogenous production of ROS and/or RNS through oxidative stress, chronically generated by inflammation [3], and also that the infection can lead to malignancy through production of bilharzial toxins [4]. In this paper, we have summarized some of the first evidence supporting an association between soluble components of the parasite *S. haematobium* and an elevated level of inflammation demonstrated by the presence of inflammatory infiltrate characterized by lymphocytes and mastocytes. In summary, we have described an animal model system that is very amenable to studies of the role of inflammation in cancer.

Further investigations using *Schistosoma haematobium* antigens and the identification of the carcinogenic component(s) may be useful to understand oncogenesis associated with this parasite.

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PAPER V

Carcinogenic ability of *Schistosoma haematobium* through oncogenic mutation of KRAS gene

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SUMMARY

Schistosoma haematobium is a parasitic flatworm that infects millions of people, mostly in the developing world, and is associated with high incidence of bladder cancer, although why is not clear. Previously, we have used CD-1 mice to show that *Schistosoma haematobium* total antigen (Sh) has a carcinogenic ability. Sh intravesically instillation induced the development of several urothelial lesions, namely nodular hyperplasia and dysplasia (LGIUN—Low Grade Intra-Urothelial Neoplasia)–after 40 weeks of treatment. These results suggested that Sh induce urothelium malignization. Bladder carcinoma frequently harbours gene mutations that constitutively activate the receptor tyrosine kinase-Ras pathway for this reason we studied activating mutations in KRAS gene. Twenty percent of the bladders with dysplasia presented a KRAS mutation in codon 12 of exon 2. We concluded from these results that the parasite extract of *S. haematobium* has carcinogenic ability possibly through oncogenic mutation of KRAS gene.

INTRODUCTION

Schistosomiasis is a common parasitic disease that ranks second only to malaria as a global cause of morbidity and mortality. It occurs in 76 tropical and subtropical countries and affects about 10% of the world's population. The prevalence of this infection remains high despite the availability of efficacious antischistosomal drugs. Individuals become infected with the parasite at an early age by exposure to the free-swimming larval stage (termed cercariae) in contaminated water [1]. Schistosomiasis is the first in prevalence among all water-borne infectious diseases and one of the main occupational risks encountered in the rural endemic areas of the world. Schistosomiasis-associated bladder cancer defines a characteristic pathological and histogenic nature that differentiates it from bladder cancer of non-schistosomal origin. It shows an increased incidence of squamous cell carcinoma as opposed to transitional cell carcinoma, a lower mean age at diagnosis, and a higher male/female ratio. Moreover, in association with schistosomiasis, cancer may originate throughout the bladder but rarely in the trigone, a frequent site of tumor origin in non-schistosomal cases [3]. Human bladder cancer is the fifth to the seventh most common cancer in Western countries. In many tropical and subtropical areas, however, it is the first among all types of cancer, mainly due to the endemic parasitism [3].

Many oncogenes participate in the development of human tumors. The most frequently activated genes are members of the ras gene family, two of which (Harvey- H) and (Kirsten-K) were first identified as being homologous to viral transforming genes, while the third (N-ras) was identified in a human neuroblastoma. The Ras gene product is a monomeric membrane-localized G protein of 21 kd that functions as a molecular switch linking receptor and nonreceptor tyrosine kinase activation to downstream cytoplasmic or nuclear events. Each mammalian cell contains at least 3 distinct ras proto-oncogenes encoding closely related, but distinct proteins. Ras genes can be activated by point mutation at codons 12, 13, 61, or 113-

117. Signal transduction by ras proteins involves hydrolysis of GTP, and activating mutations inhibit this process, locking the protein in the “on” signaling conformation (Stanley, 1995).

Activating mutations in these Ras proteins result in constitutive signaling, thereby stimulating cell proliferation and inhibiting apoptosis. Oncogenic mutations in the ras genes are present in approximately 30% of all human cancers (Adjei, 2001). The high degree of similarities shared by different Ras isoforms suggests that functional redundancy exists among these proteins, yet accumulating data support for unique roles of the Ras family members with K-ras being the most important Ras isoform (Zhang et al, 2007). Oncogenic K-ras mutations are prevalent in virtually all cancer types, making K-ras one of the most frequently mutated genes in human cancers (Zhang et al, 2007).

Mutational activation of ras protooncogenes is frequently observed in human tumors and in experimental animals (Cerutti P et al, 1994). Kras mutations have been found in bladder cancer patients (Jebar et al, 2005; Gormally et al, 2006), in transformed bladder epithelial cells (Brookes et al, 1988) and chemically-induced rat bladder tumors (Stanley, 1995).

We have previously demonstrated that normal cells treated in laboratory with *S. haematobium* total antigen, acquire cancer-like characteristics. In fact, these cells presented rapid uncontrolled division, high resistance to death and an abnormal capability to migrate [9] and, when injected into nude mice, lead to tumours development. Crucially, animals injected with non-exposed cells showed no growth [10]. We have further used CD-1 mice to show that *Schistosoma haematobium* total antigen (Sh) has a carcinogenic ability. Sh intravesically instillation induced the development of dysplasia in 70% of animals sacrificed 40 weeks after. Our results suggest that Sh induce the malignization of the urothelium (Botelho et al, 2009). Dysplasia (LGIUN— Low Grade Intra-Urothelial Neoplasia) is preneoplastic lesion that can evolutes to carcinoma *in situ*, invasive carcinoma and/or papillary carcinoma. In the present study, we addressed gene mutations that constitutively activate the receptor tyrosine kinase-Ras pathway and therefore we studied activating mutations in KRAS gene. Twenty percent of the bladders with dysplasia presented a KRAS mutation in codon 12 of exon 2. We concluded from these results that the parasite extract of *S. haematobium* may have carcinogenic ability possibly through oncogenic mutation of KRAS gene.

An improved understanding of the molecular basis underlying bladder tumor pathways and specifically schistosomiasis-associated bladder cancer is of clear importance in helping to better predict bladder tumor progression and devise optimal management strategies for this type of carcinoma.

MATERIAL AND METHODS

Animals

Eight-week-old female golden hamsters (LVG/SYR) and CD-1 mice were provided by Charles River (Barcelona, Spain). Animals spent one week being acclimated under routine laboratory conditions before starting the experiments. They did not receive any treatment prior to the study. Hamsters were kept in separated cages and mice were kept in 6 litter mates cage. 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 ± 2ºC) 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

Urine was collected from *S. haematobium*-infected individuals. The individuals were living in Angola, an endemic area for schistosomiasis. Informed consent from patients was obtained. *S. haematobium* infection was detected by microscopically observation of the eggs in the sediment of centrifuged urine. The eggs were hatched and with the resulting miracidia, snails from susceptible species (eu poria aqui o nome das espécies) were infected.

Golden hamsters were experimentally infected with 100 cercariae by tail immersion. The control animals consisted of littermates. The cercariae were obtained by shedding of snails infected with miracidia.

S. haematobium total antigen (Sh) production

Angolan strain of *S. haematobium* adult worms were collected by perfusion of the hepatic portal system of golden hamsters at seven weeks after infection with 100 cercarie. The worms were suspended in PBS and then sonicated. The protein extract was then ultracentrifuged and the protein concentration was estimated using a micro BCA protein assay reagent kit.

Experimental design

Forty mice were randomly divided into six experimental groups. Groups 1 and 3 (n=10) were Sh-instilled animals. Groups 2 (n=5) and 4 (n=5) were control animals with their bladders instilled with saline only. Groups 1 and 2 were sacrificed 20 weeks after instillation; group 3 and 4 were sacrificed 40 weeks after instillation. For the duration of the study, the mice's state of health was monitored daily.

For intravesical instillation of the parasite total antigen, 50 □g/ml of Sh was introduced directly into the urinary bladders of female mice via the urethra according to the procedure previously described [11]. Briefly, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). The bladder was catheterized via the urethra with a 24 gauge plastic intravenous cannula and the bladder mucosa was then traumatized to remove the umbrella cells of the urothelium by instilling 0.1 ml of 0.1N HCl solution for 15 seconds, neutralized with 0.1 ml of 0.1N KOH and flushed with sterile saline.

A total of 150 □l of Sh with a concentration of 50 □g/ml was instilled via the cannula. The dwell time after instillation was approximately 1 hour. The mice were turned 90 degrees every 15 minutes to facilitate whole bladder exposure to the Sh intravesically instilled. One hour

after the instillation, micturition of the mice was induced by light abdominal massage so that the duration of exposure was constant. Body temperature was maintained with a homoeothermic bandage. All the experiments described herein were carried out under aseptic conditions and performed once during this study.

All animals were sacrificed by intraperitoneal administration of sodium pentobarbital (130 mg/Kg). Complete necropsies were carefully conducted. All organs were examined macroscopically for any changes. The urinary bladders were inflated *in situ* by injection of 10% phosphate buffered formalin (150µl), ligated around the neck to maintain proper distension and then were immersed in the same solution for 12 hours. After fixation, the formalin was removed; the urinary bladder was cut into two strips, and routinely processed. Tissue sections (2 µm) from urinary bladder, spleen, liver and lung were stained with haematoxylin and eosin (HE) to characterize changes and inflammatory infiltrate.

Mutation analysis of KRAS gene

Mutational analysis of KRAS codons 12, was performed by direct sequencing.

RESULTS

Animals' general aspect

During the experimental work no animal died and all, animals exhibited normal cage activity. The weight of the animals remained normal throughout the experiment (data not shown).

Macroscopic and microscopic evaluation

The bladders from all groups showed no gross lesions. No macroscopic changes were seen in the liver, lungs or kidneys of all animals. The mean liver, kidney and bladder weights, as well as relative liver, kidney and bladder weights at the end of the study did not show differences (data not shown).

The bladder histopathological findings observed in all groups are given in Table 1 and Figures 1 and 2. Bladder histopathology of animals instilled with Sh was characterized by extensive diffuse or nodular infiltrate composed predominantly of lymphocytes admixed with variable number of *eosinophils*, and by absence of umbrella cells, nodular hyperplasia and dysplasia. The nodular hyperplasia exhibiting budlike proliferated urothelial cells extending into the lamina propria. After 20 weeks of treatment 30% of these animals displayed urothelial dysplasia a LGIUN (Low Grade Intra Urothelial Neoplasia), whereas after 40 weeks of treatment we found 70% of the bladders with this LGIUN, a non-invasive malignant flat lesion in the urothelium of these animals. Mastocytes were only found in the bladders of animals sacrificed after 40 weeks. No bladder histopathological changes were observed in animals from control groups. No microscopic changes were seen in the spleen, lung, kidney and liver in samples obtained from all animals.

Frequency of *KRAS* mutations in codon 12

Two (out of 10) of the bladders with dysplasia presented a KRAS mutation in codon 12 of exon 2 (Fig. 2). We concluded from these results that the parasite extract of S. haematobium has carcinogenic ability possibly through oncogenic mutation of KRAS gene.

DISCUSSION

Bladder cancer is a complex disease, with a multitude of genetic aberrations already uncovered, and likely more still to come. We previously demonstrated that *Schistosoma haematobium* total antigen has a carcinogenic ability in a CD-1 mice model. We showed that intravesically administration of Sh gave rise to a high incidence of urothelial dysplasia, a non-invasive malignant flat lesion, in 70% of the tested subjects after 40 weeks of treatment. In the present study, we were able to investigate this effect further and show that 20% of the dysplastic bladders presented a mutation in codon 12 of Kras gene.

It is generally recognized that the pathogenesis of malignancy involves three distinct and sequential effects on the target cells; initiation, promotion and progression. The first stage is thought to involve the formation of damage in the DNA resulting in a somatic cell mutation. The essential feature of promotion, however, is the induction of proliferation in the initiated cells by agents that are not necessarily carcinogenic per se [3]. The sequence of lesions identified, via histopathology, between initiation and promotion are designated as preneoplastic lesions and/or benign neoplasias [16]. Their transformation into malignant lesions is the last of the stages of carcinogenesis and is the most extended – it is labelled progression [17,18]. We cannot state at this point that kras mutation that we detected in dysplastic bladders induced by Sh treatment is an initiating event. The presence of this mutation could have one of two explanations: either the parasite total antigen has carcinogenic molecule(s) sufficiently genotoxic and, consistent with the mutation observed, caused direct attack on the ras gene leading to uncontrolled proliferation of the cells and causing dysplasia in the bladders treated with the parasite antigen; or the parasite antigens are only mitogenic for the urothelial cells causing increased proliferation that resulted in the kras activation via non-carcinogen-mediated pathways.

In agreement with the first explanation other researchers have well characterized the phenomenon of ras oncogene activation in a number of systems. In some cases, such as the mouse and rat mammary gland, ras activation is clearly an important initiating event, and when tumors are induced by genotoxic carcinogens the mutations observed are consistent with direct

attack by the carcinogen on the ras gene (Stanley, 1995). Also the majority of evidence taken from several animal studies suggests that ras proto-oncogene activation is an early event. This presumption is derived from detection of ras activation in benign (early) tumors of skin, lung and liver. Based upon tumor induction following a brief exposure to carcinogens that produce a chemical-specific point mutation in ras activation, researchers have suggested that activation of ras may be the initiating event in some model systems (Moronpot et al, 1995). Furthermore, the activated ras gene has been detected in many benign tumors, including mouse skin papillomas, mouse lung and liver adenomas, and basal cell and clitoral gland tumors of the rat. This implies that the activated ras was present in the cell that clonally expanded to these benign tumors. Thus, activation of the ras proto-oncogene may be the initiation event in some model systems (Stowers et al, 1987).

Few studies were carried out to investigate the role and frequency of activated ras in schistosomiasis-associated bladder cancer (Badawi, 1996). The mutations were not detected in two previous studies (Fugita et al, 1987; Ramchurren N et al, 1995). These findings indicate that the advanced stage of schistosomiasis-associated bladder cancer may not involve a high frequency of detectable mutations within the ras genes. It is known that more than 90% of the SCC in the schistosome-infected bladder were advanced (stages T3 and T4) at the time of diagnosis. However, this does not exclude the possibility that activation in ras could be an important factor in the initiation as well as promotion and progression of schistosomiasis-associated bladder cancer (Badawi, 1996).

Further studies are needed to address the functional effect of Kras mutations in schistosomiasis-associated bladder cancer.

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FIGURE LEGENDS

Figure 1: (A and B) control, (C) urothelium displaying an inflammatory nodule and a lesion with characteristics of nodular hyperplasia, (D) denudation and dysplasia, (E) inflammatory infiltrate and dysplasia, (F) inflammatory nodule and dysplasia.

Figure 2: KRAS exon 2 sequencing results. (A) control, (B) dysplastic bladder with a mutation at codon 12 (resulting in a Gly12Asp mutation).

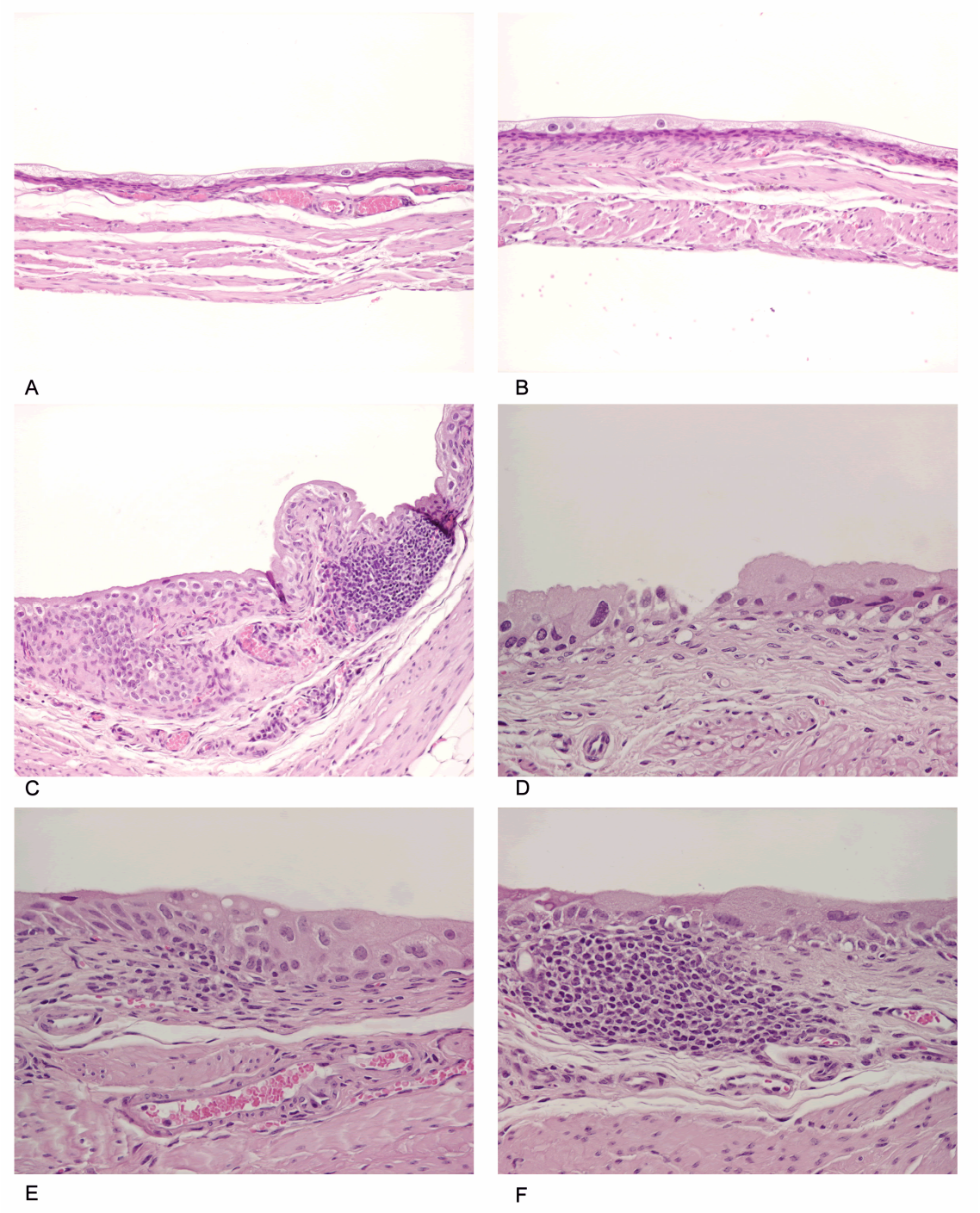


Figure 1

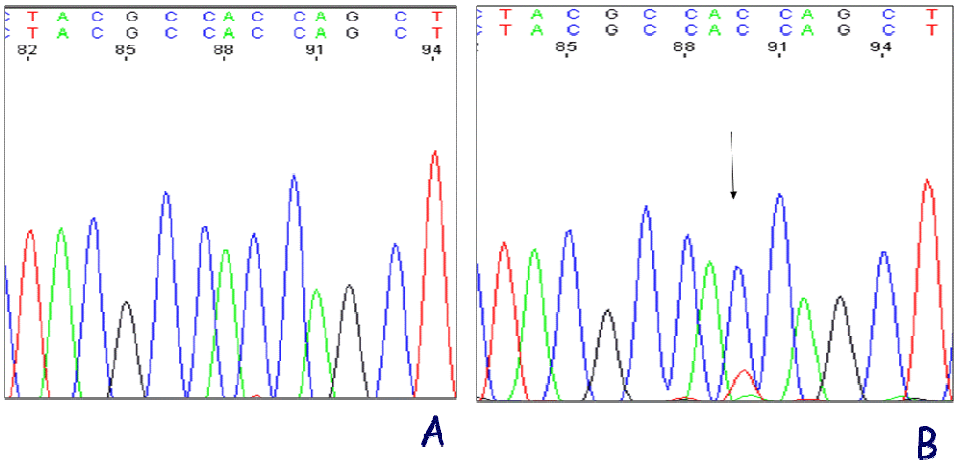


Figure 2

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Research Brief

Schistosoma haematobium and *Schistosomiasis mansoni*: Production of an estradiol-related compound detected by elisa

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ABSTRACT

Estradiol is a steroid hormone secreted principally by the ovarian follicles in vertebrate animals. We have identified the production of an estradiol-related molecule in the trematodes *Schistosoma haematobium* and *Schistosomiasis mansoni*. We show in this work that this molecule related to estradiol is present in schistosome worm extracts. The detection method ELISA specific for estradiol, revealed the expression of this estradiol-related molecule in schistosome worm extracts, but not in *Fasciola hepatica* worm extracts. Our results demonstrate for the first time the production of an estradiol-related compound by a human parasite of the genus *Schistosoma*.

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1. Background

Human schistosomes currently infect more than 200 million people in 74 countries worldwide in the endemic areas of Africa, the Caribbean, Central America, South America, eastern Asia, and the Middle East. Prevalence is thought to be rising mainly due to increasing travelers from the USA and Europe to these endemic regions for business or leisure. Wars are also known to increase the impact of schistosomiasis as demonstrated by a case recently published by our group (Vieira et al., 2007).

Hormones regulate a variety of cellular and physiological functions of organisms, such as growth, reproduction, and differentiation. The mechanisms by which host hormones act on parasites have recently been investigated, and some parasite molecules that are involved in transregulation have been identified and characterized (Escobedo et al., 2005).

In the case of schistosomes, some hormonal signals from the host seem to have a major influence on larval homing, survival, growth, and sexual maturation. Moreover, this is a 2-way dialogue and certain helminth parasites can have a particularly marked effect on the fecundity of the host (de Mendonça et al., 2000).

Human steroid hormones have yet not been identified, to our knowledge, in eukaryotic parasites. At present, only ecdysteroids have been identified in *Schistosomiasis mansoni* (Nirde et al., 1983).

While addressing schistosomiasis-induced hypogonadism by several hormones in patients infected with *S. haematobium* and *S. mansoni*, we observed an extraordinary increase in serum levels of estradiol. This molecule is a steroid hormone with a molecular mass of 272.39 Da, which circulates predominantly as a protein-bound moiety. In addition to estradiol, other natural steroidal estrogens include estrone, estriol, and their conjugates. Estrogens are hormones secreted principally by the ovarian follicles, but also by the adrenals, corpus luteum, placenta, and, in males, by the testes. Estrogenic hormones are also secreted at varying rates, with the gonadotropins of the anterior pituitary regulating secretion of estradiol and progesterone. Hypothalamic control of pituitary gonadotropin production is, in turn, influenced by plasma concentrations of estrogens and progesterones (March et al., 1979). Surprisingly, the serum levels of the pituitary gonadotropins, Luteinizing Hormone (LH), and Follicle Stimulating Hormone (FSH), in these patients remained normal. We would expect a decrease in LH and FSH due to the normal regulating feedback system observed in the endocrine system. Therefore, we hypothesized that the excess estradiol could only be external to the host. Here, we report the production of an estradiol-related molecule in *S. haematobium* and *S. mansoni* total antigen extract. To our knowledge, this is the first report identifying an estradiol-related compound as a hormone produced by a parasite.

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2. Materials and methods

2.1. Sera

Sera from 17 Schistosoma infected individuals and eight controls were obtained from the parasitology lab (INSA-Porto). The individuals were living in endemic areas (Angola, Mozambique and Guinea). All sera were selected on the basis of positive urine and fezes egg counts. The controls were living in the same endemic areas.

2.2. S. haematobium, S. mansoni, and F. haepatica total antigen production

S. haematobium and *S. mansoni* adult worms were collected by perfusion of the hepatic portal system of golden hamsters and balb/c mice respectively, at 7 weeks after infection with 100 cercarie. *Fasciola hepatica* worms were collected from the livers of infected bovines in slaughter houses. The worms were suspended in PBS and then sonicated. The protein extract was then ultracentrifuged and the protein concentration was estimated using a micro BCA protein assay reagent kit.

2.3. Animals and experimental infections

Ten golden hamsters and 10 balb/c mice were experimentally infected with 100 cercariae of *S. haematobium* and *S. mansoni*, respectively. Hamsters and mice were infected by member's extremities and tail immersion, respectively. The control animals consisted of 10 littermates. The cercariae were obtained by shedding of snails infected with miracidia.

2.4. Hormone serum levels

Estradiol, Testosterone, LH and SHBG were determined, using a solid phase competitive chemiluminescent enzyme immunoassay with the Immulite 2000 Analyser (DPC) according to the manufacturer's instructions. Briefly, the solid phase (bead) is coated with rabbit anti-hormone polyclonal antibody. The reagent contains alkaline phosphatase (bovine calf intestine) conjugated to the hormone analyzed. The hormone-enzyme conjugate competes with the tested hormone in the patient sample for limited antibody-binding sites on the bead. The excess sample and reagent are removed by a centrifugal wash. Finally, chemiluminescent substrate is added to the bead and signal is generated in proportion to the bound enzyme.

2.5. Estradiol by ELISA

Estradiol levels from in vitro assays were determined using ELISA (Calbiotech, CA, USA) according to the manufacturer's instructions. Briefly, the E2 ELISA is based on the principle of competitive binding between E2 in the test specimen and E2-HRP conjugate for a constant amount of rabbit anti-Estradiol. In the incubation, goat anti-rabbit IgG-coated wells are incubated with E2 standards, controls, patient samples, Estradiol-HRP Conjugate Reagent and rabbit anti-Estradiol reagent at room temperature for 90 min. During the incubation, a fixed amount of HRP-labeled E2 competes with the endogenous E2 in the standard, sample, or quality control serum for a fixed number of binding sites of the specific E2 antibody. E2 peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of E2 in the specimen increases. Unbound E2 peroxidase conjugate is then removed and the wells washed. Next, a solution of TMB Reagent is added and incubated at room temperature for 20 min, resulting in the development of blue color. The color development is stopped with the addition of stop solution, and the absorbance is measured spectrophotometrically at 450 nm. A standard curve is obtained by plotting the concentration of the standard versus the absorbance.

2.6. Statistical analysis

Statistical tests were performed using the WassarStats: web site for Statistical Computation software. The values of Estradiol for various groups were compared using Mann–Whitney non-parametric test for the significance of the difference between the distributions of two independent samples.

3. Results and discussion

In a series of patients infected with *S. haematobium*, we attempted to identify and quantify the sex hormones estradiol (E2), testosterone and luteinizing stimulating hormone (LH) in their sera by ECLIA (electrochemoluminescence) (Immulite 2000 Analyser, DPC; Terrytown, New York) (Table 1). The individuals were living in endemic area and all sera were selected on the basis of positive egg counts. We observed that in all of these patients, serum levels of estradiol were significantly elevated in comparison with non-infected controls living in the same endemic areas. One of the patients, a 4-yr-old girl, had E2 levels similar to those at puberty. The other hormones presented normal levels. The fact that LH was normal in these patients is surprising, and contradicts the normal feedback effect of the endocrine system, in which LH should lower the excess E2. The increase in E2 levels is not attributable to a change in Sex Hormone Binding Globulin (SHBG) levels (data not shown).

We hypothesized that the elevated levels of estradiol observed in *Schistosoma*-infected patients were produced by the parasite itself and, therefore, we analyzed the total antigen extract of the parasites. We observed the expression of an estradiol-related molecule. For this purpose, adult *S. haematobium* and *S. mansoni* were recovered by total perfusion of the hepatic portal system of golden hamsters at 7 weeks post- infection with 100 cercarie. The worms were washed three times in PBS, suspended in PBS, and then sonicated. The protein extract was centrifuged (12,000*g*) and the protein concentration was estimated using a micro BCA protein assay reagent kit (Rockford, Illinois). In all experiments, we used 400 μg/ml of total antigen. The estradiol production analyzed by ELISA in the *S. haematobium* total antigen was 15 pg/ml of antigen extract. We found that *S. mansoni* also produces this estradiol-related molecule, although in a smaller concentration (13 pg/ml). This is the first time that a related compound of a human hormone has been identified in a parasite.

We also tested *F. hepatica* via ELISA and found that estradiol is half the concentration in schistosomes and below the detection limit of the method used (7.5 pg/ml).

Our results clearly show the production of a related molecule of estradiol by two species of *Schistosoma*. This production seems to be genus specific since adults of *F. hepatica* do not produce the same molecule.

The estradiol-related compound production in *Schistosoma* species is not surprising. Several authors have attempted and succeeded in identifying hormones in this group of parasites. For example, Nirde et al. (1983) demonstrated the presence of the insect molting hormones, ecdysone and 20 hydroxyecdysone, in *S. mansoni* by the combined use of radioimmunoassay and high pressure liquid chromatography (HPLC). These authors thus established for the first time that *S. mansoni* synthesizes steroid hormones. Later, the same team of researchers (Nirde et al., 1984) demonstrated

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Table 1
Sera of *Schistosoma*-infected patients and controls were subject to analysis by ECLIA for the sex steroids estradiol (E2), testosterone, luteinizing hormone (LH). Typical reference values (range) are shown for sex and age.

Group	Sex	Age (yr)	E2 (pg/ml)	Range	Testosterone (ng/dl)	Range	LH (mUI/ml)	Range
1 (Sh)	Female	4	62.8[a]	0–22	<15.0	2–10	0.114	<2.5
1 (Sh)	Male	12	30.8	0–25	77.5	5–500	1.79	0.2–8.0
1 (Sh)	Male	14	79.8	0–25	363	5–500	1.89	0.2–8.0
1 (Sh)	Male	17	45.7	0–25	724	>200	5.89	1.4–7.7
1 (Sh)	Male	17	31.9	0–25	535	>200	7.65	1.4–7.7
1 (Sh)	Male	20	68.3	<56.0	982	262–1593	2.87	1.4–7.7
1 (Sh)	Male	22	65.1	<56.0	1225.2	262–1593	3.61	1.4–7.7
1 (Sh)	Male	21	67.6	<56.0	266.1	262–1593	4.9	1.4–7.7
2 (Sm)	Male	30	76.1	<56.0	1027	262–1593	2.48	1.4–7.7
2 (Sm)	Male	28	104.3	<56.0	923.9	262–1593	5.58	1.4–7.7
2 (Sm)	Male	25	77.1	<56.0	1084.3	262–1593	3.00	1.4–7.7
2 (Sm)	Male	32	75.8	<56.0	1499	262–1593	1.60	1.4–7.7
2 (Sm)	Male	20	152.6	<56.0	1488.1	262–1593	1.90	1.4–7.7
2 (Sm)	Male	35	84.9	<56.0	683.4	262–1593	N.D.	1.4–7.7
2 (Sm)	Male	24	88.21	<56.0	740.4	262–1593	4.12	1.4–7.7
2 (Sm)	Male	25	58.1	<56.0	698.7	262–1593	3.84	1.4–7.7
2 (Sm)	Male	23	59.2	<56.0	1038.2	262–1593	1.86	1.4–7.7
3 (Ctrl)	Male	29	44.7	<56.0	422.8	262–1593	2.72	1.4–7.7
3 (Ctrl)	Male	24	39.3	<56.0	385.1	262–1593	7.07	1.4–7.7
3 (Ctrl)	Male	26	48.8	<56.0	642	262–1593	1.79	1.4–7.7
3 (Ctrl)	Male	28	49.3	<56.0	548.6	262–1593	1.19	1.4–7.7
3 (Ctrl)	Male	28	38.6	<56.0	464.5	262–1593	1.50	1.4–7.7
3 (Ctrl)	Male	22	35.9	<56.0	379.3	262–1593	3.16	1.4–7.7
3 (Ctrl)	Male	24	50.1	<56.0	702.1	262–1593	N.D.	1.4–7.7
3 (Ctrl)	Male	27	43.5	<56.0	433.7	262–1593	1.7	1.4–7.7

Sh, *S. haematobium*-infected patients; Sm, *S. mansoni*-infected patients; Ctrl, Controls; N.D., Not determined.
[a] ($P = <0.0001$, Mann–Whitney test infected vs. control individuals).

that these molecules could be used for parasite diagnosis. Estradiol has by far a much wider use than ecdysteroids, as it is currently used in many clinical chemistry labs for a variety of diagnostic uses.

The purpose of estradiol in these parasites could be very wide. In humans, estradiol is involved in several cellular and molecular mechanisms. For example, estradiol is known to protect endothelial cells from apoptosis (Spyridopoulos et al., 1997). Since adult schistosomes live with their ventral suckers attached to the endothelium feeding on circulating blood, they require stabilization of endothelial cells to guarantee their survival. Estradiol also has important regulatory functions in promoting leukocyte-endothelial cell interactions (Cid et al., 1994). Inflammation is a fundamental process in the progression of the life cycle of schistosomes; without it there is no granuloma formation and, therefore, there would be no release of the eggs to the exterior of the host. Adhesion of leukocytes to endothelial cells is a critical step in the development of acute and chronic inflammatory lesions. Estradiol also increases endothelial monolayer permeability (Groten et al., 2005). Since the eggs of schistosomes must cross the capillary wall, the permeability of this wall is a very important step in the release of eggs to the lumen of the intestine or the bladder (Loeffler et al., 2002).

The significance of our findings is supported by the knowledge that schistosomes have estradiol receptors (Barrabes et al., 1986). In mammals, estradiol regulates diverse physiological processes in reproductive tissues and in mammary, cardiovascular, bone, liver, and brain tissues. The effects of estradiol are mediated via its receptors ER, which are members of the nuclear receptor superfamily of ligand-activated transcription factors that control these physiological processes, in large part through the regulation of gene transcription. A molecule able to bind estradiol has been previously described in schistosomes. It could be used by the parasite, as a likely mechanism for the protective effect of this hormone in infected mice and hamsters (Barrabes et al., 1986). Further studies are necessary to confirm the homology between mammal ER and the receptor described by Barrabes and collaborators (1986).

Recent experimental evidence suggests that schistosomes can not only evade immune responses actively, but also exploit the hormonal microenvironment within the host to favor their establishment, growth, and reproduction (Escobedo et al., 2005). It is generally believed that infection by schistosomes leads to modifications in the hormonal status of their hosts and that this is the result of a secondary reaction of the host to infection, one that is not directly mediated by parasite-derived factors (de Mendonça et al., 2000).

In the light of the results presented here, we demonstrate for the first time a molecule produced by these parasites. Further studies are necessary to identify and characterize this related estradiol molecule production by schistosomes, and assert the importance of this new molecule to the life cycle of the parasite. Blocking the binding of this molecule with its receptor, with the use of anti-hormonal therapy like ICI 182,780, a potent antiestrogen with the ability to inhibit and downregulating ER, could be explored as an alternative therapy to schistosomiasis.

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PAPER VII

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Schistosoma haematobium: Identification of new estrogenic molecules with estradiol antagonistic activity and ability to inactivate estrogen receptor in mammalian cells

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ABSTRACT

We have previously identified the expression of an estradiol (E2)-related molecule by *Schistosoma haematobium* total antigen (Sh). We now show that this molecule has an antagonistic effect of estradiol in vitro. Our results are consistent with the existence of an estrogenic molecule that antagonizes the activity of estradiol. We found evidence for this molecule as we identified and characterized by mass spectrometry new estrogenic molecules previously unknown, present in schistosome worm extracts and sera of schistosome infected individuals. We also show that Sh is able to interact in vitro with estrogen receptor (ER), explaining how host endocrine system can favor the establishment of schistosomes. These findings highlight the exploitation of the host endocrine system by schistosomes and represent an additional regulatory component of schistosome development that defines a novel paradigm enabling host–parasite interactions. The identification of these molecules opens new ways for the development of alternative drugs to treat schistosomiasis.

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1. Introduction

Despite affecting 200 million people, schistosomiasis remains a neglected disease. There are two main reasons for this: the availability of an effective and cheap drug, praziquantel, has contributed to the wrong perception that chemotherapy would represent the final solution for the control of schistosomiasis, and the disability adjusted life years as a measure of the impact of disease has been under evaluated because of rebound morbidity following chemotherapeutic intervention (Capron et al., 2005). Prevalence is thought to be rising mainly due to increasing travelers from the USA and Europe to these endemic regions for business or leisure. Wars are also known to increase the impact of schistosomiasis as demonstrated by a case recently published by our group (Vieira et al., 2007).

The mechanisms by which host hormones act on parasites have recently been investigated, and some parasite molecules that are involved in transregulation have been identified and characterized. In the case of schistosomes, hormonal signals from the host seem to have a major influence on larval homing, survival, growth and sexual maturation (Mendonça et al., 2000; Escobedo et al., 2005). In the case of schistosomes, some hormonal signals from the host seem to have a major influence on larval homing, survival, growth, and sexual maturation. Moreover, this is a 2-way dialogue and certain helminth parasites can have a particularly marked effect on the fecundity of the host (Mendonça et al., 2000).

Nuclear hormone receptors comprise a large superfamily of transcription factors whose activity is under hormonal control. These receptors are characterized by a central DNA-binding domain, which interacts with specific hormone response elements located near the target gene promoter, and by two distinct activation function (AF) domains that contribute to the transcriptional activity of these receptors (Delage-Mourroux et al., 2000).

The estrogen receptor (ER), a member of the steroid receptor family, mediates the stimulatory effects of estrogens and the

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inhibitory effects of antiestrogens in estrogen target cells (Martini et al., 2000). Estrogen receptor (ER), mediates the biological effects of estrogens in a variety of target tissues (Delage-Mourroux et al., 2000) and ER-regulated genes are involved in many biological processes, including cell growth and differentiation, morphogenesis, and programmed cell death (Martini et al., 2000). The bound estrogen receptor stimulates transcription via interaction with the estrogen response element (ERE) in the nucleus (Catherino and Jordan, 1995).

We recently reported the expression of an estradiol-like molecule by a trematode parasite *Schistosoma haematobium* (Botelho et al., 2009a). To better understand the mechanism by which this molecule works, we transfected CHO cells with an expression plasmid containing an ERE linked to a luciferase and treated the cells with *S. haematobium* total antigen (Sh). In this way, as we show in this paper, we identified interaction of Sh with the ER. Intriguingly, we find that the estradiol-like molecule is an antagonist of estradiol, repressing the transcriptional activity of the ER. To better characterize this molecule we recurred the technique of mass spectrometry and developed a new method for the detection of estrogenic molecules in biologic samples. In this way, as we show in this paper, we identified new estrogenic molecules in *S. haematobium* total antigen as well as in the serum of infected individuals with this parasitic disease that seem to be specific of this parasite.

Elucidating the role of estrogens signaling pathway during *S. haematobium* infection will determine the use of new drug targets to treat this disease.

2. Materials and methods

2.1. Animals

Eight-week-old female golden hamsters (LVG/SYR) were provided by Charles River (Barcelona, Spain). Animals spent one week being acclimated under routine laboratory conditions before starting the experiments. They did not receive any treatment prior to the study. Hamsters were kept in separated cages and fed standard balanced food and water ad libitum. All the animals were raised and 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 to 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).

2.2. Parasites

Schistosoma haematobium (Angolan strain) life cycle was maintained through successive passages in laboratory-raised Bulinus truncatus as invertebrate hosts and golden hamsters as vertebrate hosts. Cercariae of *S. haematobium* were obtained from infected snails by the use of artificial light.

2.3. Experimental infections

Urine was collected from *S. haematobium*-infected individuals. The individuals were living in Angola, an endemic area for schistosomiasis. Following instruction in midstream urine collection, urine samples were then collected from each individual. *S. haematobium* infection was detected by microscopically observation of the eggs in the sediment of centrifuged urine. Informed consent from patients was obtained. The eggs were hatched and with the resulting miracidia, snails from susceptible species were infected. Golden hamsters were experimentally infected with 100 cercariae by tail immersion. The control animals consisted of littermates. The cercariae were obtained by shedding of snails infected with miracidia.

2.4. *Schistosoma haematobium* total antigen production

Schistosoma haematobium adult worms were collected by perfusion of the hepatic portal system of golden hamsters at seven weeks after infection with 100 cercariae. The worms were suspended in PBS and then sonicated. The protein extract was then ultracentrifuged (20,000 RPM) and the protein concentration was estimated using a micro BCA protein assay reagent kit.

2.5. Sera

Sera from *S. haematobium*-infected individuals were obtained from CIBP (INSA-Porto). All sera were selected on the basis of positive urine egg counts for *S. haematobium*.

2.6. Chemicals and materials

CHO cells were cultured in CHO medium (Sigma Aldrich, Portugal) and MCF-7 cells were cultured in RPMI 1640 medium phenol red free (Sigma Aldrich, Portugal), with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, purchased in Invitrogen Life Technologies (Paisley, Scotland, UK). Estradiol (E2) was obtained from Sigma Aldrich (Portugal) and the antiestrogen ICI 182,780 (ICI) was kindly provided by AstraZeneca (Portugal), both chemicals dissolved in 100% ethanol and added to cell culture medium.

2.7. Plasmids

Plasmids were used to transform DH5α *Escherichia coli*. Individual colonies were isolated and plasmid miniprep was done using the Promega Miniprep Kit. The desired plasmid was amplified and isolated using the Qiagen maxiprep kit. The estrogen-responsive reporter plasmid, pERE-Luc, was kindly provided by Ming Tsai and Bert O'Malley (Baylor College of Medicine, Houston, TX) (Nawaz et al., 1999).

2.8. Cell culture

CHO cells and MCF-7 cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere. Cells were passaged every 5 days. Before treatments cells were serum-starved for 16 h. The cells were treated with Sh antigen for 24 h in serum-free conditions.

2.9. Estradiol assay

CHO cells were cultured in 0.3 ml CHO media in 24-well flat-bottomed plates. Cultures were seeded at 5×10^4 cells/well and allowed to attach overnight. Cells were then treated with increasing concentrations of Sh for 24, 48 and 72 h. Estradiol in the supernatant of CHO cells was determined using electrochemiluminescence (ECLIA) with the Immulite 2000 Analyser (DPC) and ELISA (Calbiotech) according to the manufacturer's instructions.

2.10. Lactoferrin assay

MCF-7 cells were cultured in 0.3 ml RPMI medium in 24-well flat-bottomed plates. Cultures were seeded at 5×10^4 cells/well and allowed to attach overnight. Cells were then treated with 50 μg/ml of Sh for 24 h. Lactoferrin in the supernatant of MCF-7 cells was determined using ELISA with an antibody specific for lac-

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toferrin (Hycult, Netherlands) according to the manufacturer's instructions.

2.11. Isolation of RNA

At defined time intervals MCF-7 cultured cells were harvested in Tripure reagent (Roche, Portugal). Total RNA was isolated from the cell material according to the manufacture instructions. RNA recovery and quality were checked by measuring the optical density ratio (260/280 nm) and its quantification was determined also by optical density (Nanodrop).

2.12. Real-Time PCR

Real-Time PCR was performed in MCF-7 cultures. For this purpose cDNA was obtained using the Promega cDNA kit. For each sample, 1.0 μg of RNA was reverse transcribed in a reaction volume of 20 μl in the presence of 10 mM deoxynucleotide triphosphate and 2 μl RT-PCR enzyme mix. Taqman GeneExpression assays (Applied biosystems) were used for gene-specific primers and probes for ER alpha (ESR1, PE Applied Biosystems, USA) and ER beta (ESR2, PE Applied Biosystems, USA). Both target gene transcripts were normalized to the endogenous control GAPDH mRNA (PE Applied Biosystems, USA) content and to the control sample. Quantifications were done in triplicate in 7500 Real-Time PCR System (PE Applied Biosystems, USA).

2.13. Transient transfection

CHO cells were plated for transfection in 24-well plate and incubated for 24 h at 37 °C with 5% CO_2 the day before transfection. The cells were transfected with 0.75 μg ERE-Luc plasmid per well and 0.3 μg pCMV-β (which constitutively produces β-galactosidase) for normalization. Cells were transfected by using Lipofectamine (Invitrogen, Carlsbad, California, USA) according to the manufacturer's recommended guidelines. Cells were washed and fed with CHO medium and subsequently treated with 50 μg/ml of Sh, 10^{-9} E2 and 10^{-9} M ICI. Concentrations for E2 and ICI used were based on previous studies (Soares et al., 2003). As a control, cells were treated with vehicle alone (ethanol). After 24 h, cells were harvested and cell extract were prepared for luciferase assay. Luciferase activity was determined using an enhanced luciferase assay kit (BD Biosciences, USA) in a scintillation counter (Amersham Biosciences, USA). Cell extracts were assayed for β-galactosidase activity as described previously (MacGregor and Caskey, 1989), and the luciferase units were normalized to β-galactosidase activity from the cotransfected pCMV-β internal reference plasmid.

2.14. Mass spectrometry analysis

LC–MS analysis were carried out using a Thermo Finnigan Surveyor LCQ DECA XP MAX quadrupole ion trap mass spectrometer, utilizing electrospray ionization, coupled to a LC of Thermo Finnigan. UV detection was achieved at 280 nm and the column was a Merck Purospher STAR RP-18e 125 cm × 4.6 (3 μm), equipped with a Merck Lichrocart pre-column (Merck, Germany).

The mobile phases consisted of water/acetonitrile/acetic acid mixtures, 95:5:0.5 for A and 45:55:0.5 for B. The following gradient was 0–5 min, 100% A; 5–10 min, linear gradient from 100% to 80% A; 10–15 min, 80% A; 15–50 min, linear gradient from 80% to 40% A; 50–65 min, 40% A; 65–75 min, linear gradient from 40% A to 100% B. The flow rate was 0.5 mL/min and for MS^2 experiments, the collision energy was optimized at 30%.

2.15. Statistical analysis

Data were expressed as mean ± SD. *t*-Test was used to assess the statistical significance of differences. $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Schistosoma haematobium* total antigen decreases estradiol production of CHO cells in vitro

Since we discovered that Sh expresses a molecule analogue to estradiol (Botelho et al., 2009a), we developed an assay to confirm the estradiol-like effect on estrogen responsive cells. Here we evaluate the production of estradiol by these cells, since estradiol production is a target of estradiol itself. For this purpose, CHO cells were seeded on 96 well plates, starved ON, treated with increasing concentrations of *S. haematobium* total antigen (Sh) for 24 h, cultured for 24, 48 and 72 h and their supernatants analyzed by ECLIA. Surprisingly, the curve shows that cells treated with Sh at a concentration of 50 μg/ml or higher decreased significantly estradiol production in comparison to control cells (Fig. 1).

3.2. *Schistosoma haematobium* total antigen is an antagonist of estradiol

To further elucidate the previous results, we analyzed the effects of estradiol (E2), ICI 182,780 (ICI) and Sh on estradiol production in CHO cells. Serum-starved cells in six-well plates were treated with 10^{-9} M E2, 10^{-9} M ICI or 50 μg/ml of Sh for 24 h. Supernatants were analyzed by ELISA. We observed, as expected, that ICI reverts estrogen release to the supernatant (E2 + ICI compared to E2 bars). Surprisingly, Sh decreases significantly estradiol level in supernatant to a higher extent than ICI. These findings demonstrate an antagonist effect of estradiol. Remarkably, co-culturing Sh with ICI reverts the decrease of estradiol induced by Sh, demonstrating that this effect is probably mediated by estrogen receptor (Fig. 2).

3.3. *Schistosoma haematobium* total antigen reduces the production of lactoferrin in the supernatant of MCF-7 cells

Lactoferrin is a target gene of estrogen receptor and can be a useful marker of estrogen action on estrogen responsive cells like MCF-7. Therefore MCF-7 cells were seeded on 96 well plates, starved ON, treated with 50 μg/ml of Sh for 24 h, cultured for 24 and 48 h, and the supernatants analyzed by ELISA for lactoferrin (Fig. 3). Bars show that Sh-treated cells produced less lactoferrin than control cells. In addition, incubation with ICI or Sh + ICI also led to identical decrease in lactoferin secretion ($^*p < 0.05$ vs Con-

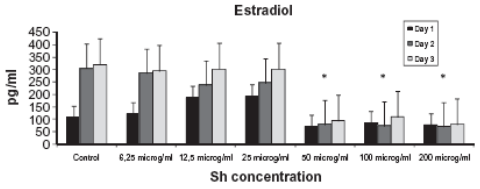


Fig. 1. Estradiol production in culture of *S. haematobium* total antigen treated CHO cells. The experiments were done in triplicate ($^*p < 0.01$; 50, 100 and 200 μg/ml vs. control) for days 1, 2 and 3. We used the concentration of 50 μg/ml of Sh for the subsequent assays.

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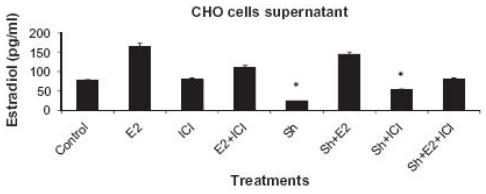


Fig. 2. Estradiol production in supernatant of CHO cells. Cells were treated with 10–9 M of E2, 10–9 M of ICI and 50 mg/ml of Sh. Data represent means ± SEM from three independent experiments (*$p < 0.05$ Sh vs. control).

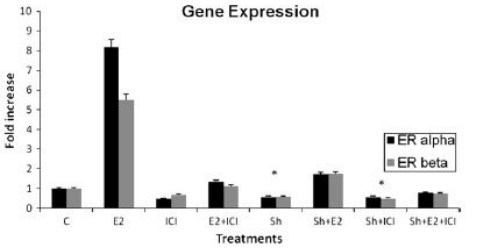


Fig. 4. Real-Time PCR for ER alpha and ER beta in MCF-7 cells. Bars correspond to fold increase of ER alpha and ER beta gene expression after normalization to endogenous control gene GAPDH and control sample. Results are representative of three independent experiments. Statistically significant differences in ER alpha and ER beta gene expression were found between Sh-treated and vehicle-treated MCF-7 cells (*$p < 0.05$ vs. control).

trols).These results suggest that Sh treatment represses estrogen receptor activity.

3.4. *Schistosoma haematobium* total antigen down-regulates ER alpha and ER beta

To confirm the involvement of estrogen receptor, expression of ER alpha and ER beta were analyzed in MCF-7 cells after estradiol, ICI and Sh treatment. Real-Time PCR analysis for ER alpha, ER beta, and endogenous control gene GADPH were carried out by using RNA derived from either vehicle-treated (control), estradiol-treated (E2), ICI-treated (ICI) or Sh-treated in MCF-7 cell cultures. Incubation with estrogen clearly increased both ERalpha and ER beta expression (Fig. 4). Furthermore, both ER isoforms were down-regulated by Sh treatment in this cell culture, either alone or in the presence of ICI in comparison to control (Fig. 4). Interestingly, Sh incubation extensively reduced ER alpha and ER beta expression, even in the presence of estrogen (*$p < 0.05$ Sh + E2 vs. E2).

3.5. *Schistosoma haematobium* repression of ER transcriptional activity

Nuclear hormone receptors regulate gene expression by interacting directly with DNA response elements. To confirm Sh-mediated down-regulation of ER we carried out transfection of CHO cells with a reporter gene construct containing an estrogen response element (ERE). As shown in Fig. 5, Sh effectively reduced the transcriptional activity of the ER. When cells are treated simultaneously with Sh and ICI there is a synergistic effect in the reduction of luciferase activity. Sh was also able to significantly reduce ER transcriptional activity in the presence of E2 (Fig. 5) as revealed by comparing Sh + E2 with E2 results.

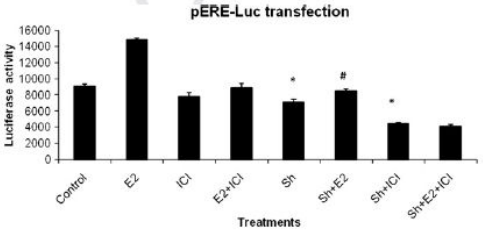


Fig. 5. Role of estradiol on activation of estrogen responsive element (ERE) was assessed by luciferase assay. CHO cells were transfected with a pGL3 reporter construct containing an ERE linked to luciferase, and incubated with 10^{-9} M E2, 10^{-9} M ICI or 50 μg/ml Sh afterwards. Reporter gene transactivation was evaluated 2 days after transfection. Luciferase activity induction was measured. Incubation with Sh or in the presence of ICI resulted in a statistically significant decrease in luciferase activity compared to control (*$p < 0.05$ Sh vs. control; Sh + ICI vs. control). Co-incubation of Sh together with E2 further reduced E2 effects on ER transcription activity (#). Bars represent mean values of three distinct assays. Error bars represent SD. Experiments were performed in triplicate.

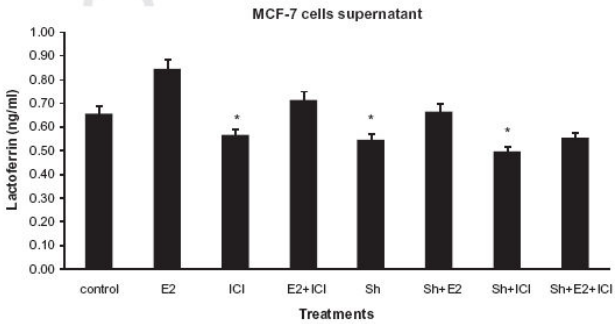


Fig. 3. Lactoferrin production in supernatant of MCF-7 cells. Cells were treated 50 μg/ml of Sh. Data represent means ± SEM from three independent experiments (*$p < 0.05$ Sh vs. control; ICI vs. control; and Sh + ICI vs. control).

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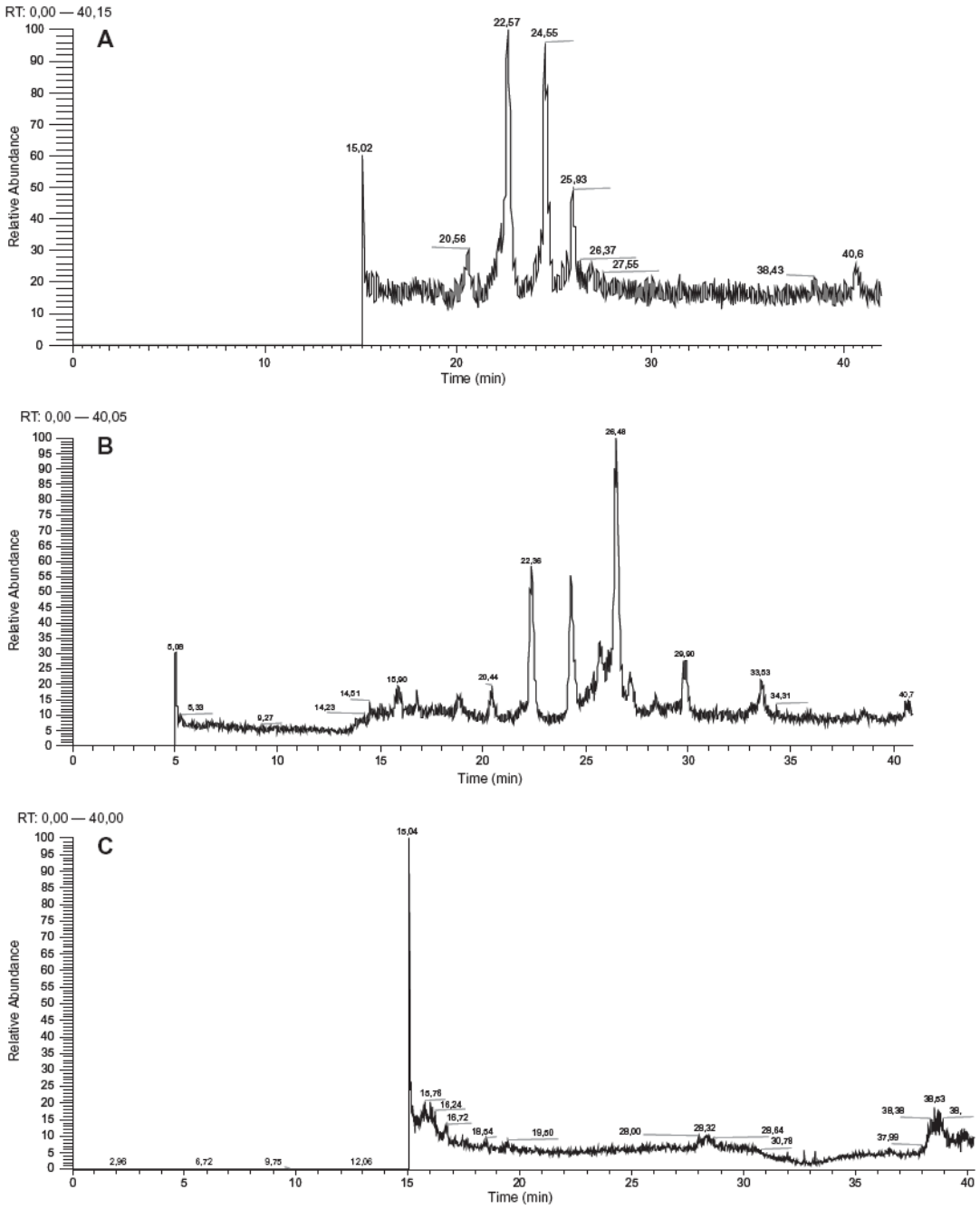


Fig. 6. LC–ESI–MS analysis of schistosoma extract (A), serum from a schistosoma-infected individual (B), and serum from a healthy donor (C). MS spectra of main components in samples analyzed are given in Supporting Information.

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Table 1
Retention times and *m/z* values for the four main components identified by LC–MS analysis of schistosoma samples.

Component	Retention time (min)	"Duplicated" *m/z* peaks detected on negative mode
1	20	610/624
2	22	723/737
3	24	836/850
4	26	949/963

3.6. Identification of new estrogenic molecules in *S. haematobium* total antigen

Liquid chromatography coupled to electrospray ionization mass spectrometry (LC–ESI–MS) was used to provide both a clear distinction between different samples and a structural identification of their main components. Fig. 6 depicts chromatograms obtained for biological samples by LC–ESI–MS. As seen, both the schistosoma extract (Fig. 6A) and the plasma from a schistosoma-infected individual (Fig. 6B) present four main peaks with retention times of approximately 20, 22, 24 and 26 min. Interestingly, these four peaks were not observed for the plasma from a healthy donor (Fig. 6C). Mass spectral analysis, run in the negative mode (i.e., species detected as [M-H]-ions), of the four main components present in both schistosoma samples revealed that those were species with molecular weights considerably higher than that of estradiol (Table 1). Moreover, the corresponding *m/z* peaks seemed duplicated (see MS spectra on Supporting Information), as each component was invariably related to two MS peaks, always detected at *m/z* ([M-H]-) and *m/z* ([M-H]-) + 14. Species detected after MS^2 fragmentation of components **1**–**4**, as illustrated in Fig. 7 for component **2** – is the most intense in the schistosoma extract – gave further support to our previous structural assignment for the four components.

4. Discussion

The transcriptional activity of ER is now well described to be negatively regulated by *S. haematobium*. The present studies document that Sh represses the activation of ER-induced estrogen responsive element. We also attempted to identify the molecule(s) in the parasite extract responsible for this effect.

The studies presented in this work indicate that Sh expresses estrogenic molecules that interact with ER by an intriguing mechanism, by down-regulating and repressing ER transcriptional activity. Our experiments show that there is a direct interaction of Sh, with ER and that this interaction is ligand-dependent. The ability of Sh to decrease transcriptional activity by the ER was observed with a reporter gene construct containing an ERE.

In this manuscript, we present data suggesting that Sh modulates the concentration of ER transcripts in the estrogen responsive MCF-7 cells, by promoting its down-regulation in a similar way as the estrogen inhibitor ICI. The hypothesis that this estrogen antagonistic effect observed in MCF-7 cells could be due to contamination of Sh extract by host animal cells that eventually would interfere with estrogen signaling was ruled out by the procedure used for Sh isolation. Worms were obtained by perfusion of golden hamster hepatic portal system, washed in saline solution, counted and sonicated in order to obtain the antigen. In addition, Sh was frozen. Therefore, the whole procedure would completely destroy host cell contamination. We used Real-Time PCR to determine the expression of ER alpha and ER beta mRNA MCF-7 cells treated with Sh, E2 and ICI in comparison to vehicle-treated control cells. Sh clearly down-regulates ER alpha and ER beta. The estrogen receptor (ER) exists in two isoforms ER alpha and ER beta with a different distribution in the body and different functions, which are not clearly identified yet (Zimmermann and von Angerer, 2007). Physiological effects of estrogen are mediated through both ER alpha and beta. Importantly, ER alpha- and ER beta-dependent pathways regulate distinct and largely nonoverlapping sets of genes. Whereas ER alpha is essential for most of the estrogen-mediated increase in gene expression, ER beta mediates the large majority (nearly 90%) of estrogen-mediated decreases in gene expression (O'Lone et al., 2007). Additionally, it is possible that Sh-induced ER alpha and beta down-regulation serves to control physiological responses in estrogen target tissues, which ultimately serves to limit the expression of estrogen responsive genes by the parasite.

Interestingly, Sh which enhances the inhibitory effectiveness of antiestrogens (Figs. 4 and 5), also moderates the activity of estrogens because Sh is recruited to the ER even in the presence of estradiol, exerting its antagonist actions. Therefore our findings imply that Sh dampens the stimulatory response to estradiol.

Our results are consistent with the existence of an estrogenic molecule that antagonizes the activity of estradiol. We found evidence for this molecule as we identified and characterized by mass spectrometry new estrogenic molecules previously unknown, present in schistosome worm extracts and sera of schistosome infected individuals. The detection method developed by our group by LC–MS analysis specific for estrogenic molecules, revealed that these new estrogens are present in schistosome worm extracts. At the light of these findings, we believe that the species detected on the LC–MS analysis of both the schistosoma extract and the plasma of a schistosoma-infected human were formed by reactions of estrogen-quinones with DNA. Such reactions might have occurred directly via Michael addition or indirectly through generation of reactive oxygen species (ROS) and led to structures (Scheme 2) compatible with the *m/z* values observed (Table 1). To confirm this hypothesis, and given our previous experience in tandem-MS studies to establish fragmentation mechanisms involving compounds relevant in other parasitic diseases (Vale et al., 2008a,b, 2009), we decided to further analyse the serum from a schistosoma-infected individual by LC–MS/MS. Species detected after MS^2 fragmentation of components **1**–**4**, as illustrated in Fig. 7 for component **2** – is the most intense in the schistosoma extract – gave further support to our previous structural assignment for the four components. We chose component **2** because is the most intense with a relative abundance of 100%. Indeed, MS^2 fragmentation of component **2** led to five species that were detected in the negative mode at *m/z* values of ca. 595, 621, 677, 695 and 720. These *m/z* values are compatible with fragments structures as those shown on Scheme 3, all of which can be easily derived from the parental structure **2**. Identical analyses was done for the remaining three components, **1**, 3 and **4**, whose MS^2 spectra are given in the Supporting Information and also show fragmentation patterns that confirm structures above proposed for those components.

An interpretation of the above findings was built at the light of well-established metabolic pathways for estrogens. It is known from the literature that estradiol is mainly metabolised by (i) oxidation of the hydroxyl function at the C17 position (via steroid dehydrogenase) to yield estrone, and (ii) hydroxylation via cytochrome P450 enzymes, occurring preferentially on C2, C4 and C16 (Bolton et al., 1998; Rathahao et al., 2000). When hydroxylation takes place on the steroid aromatic ring A, catechol-estrogens are produced (Iverson et al., 1996). Similar transformations can easily explain the fact that, on schistosoma samples, each component was associated to two *m/z* peaks always differing by 14 a.m.u. In fact, hydroxylation of both C-2 and C-3 on a steroid ring and further oxidation into an estradiol-2,3-quinone (Scheme 1), a phenomenon well described by Iverson and collaborators (1996), would lead to a mass increase of precisely 14 a.m.u.

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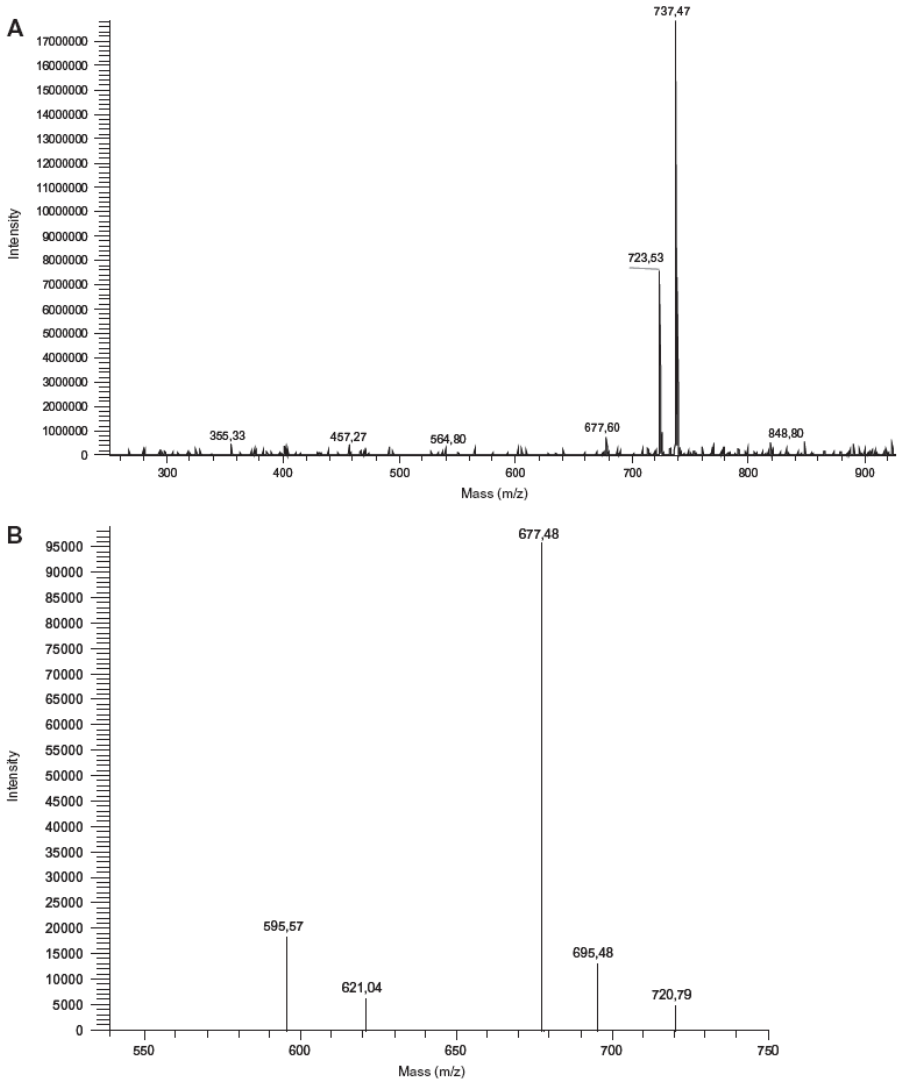
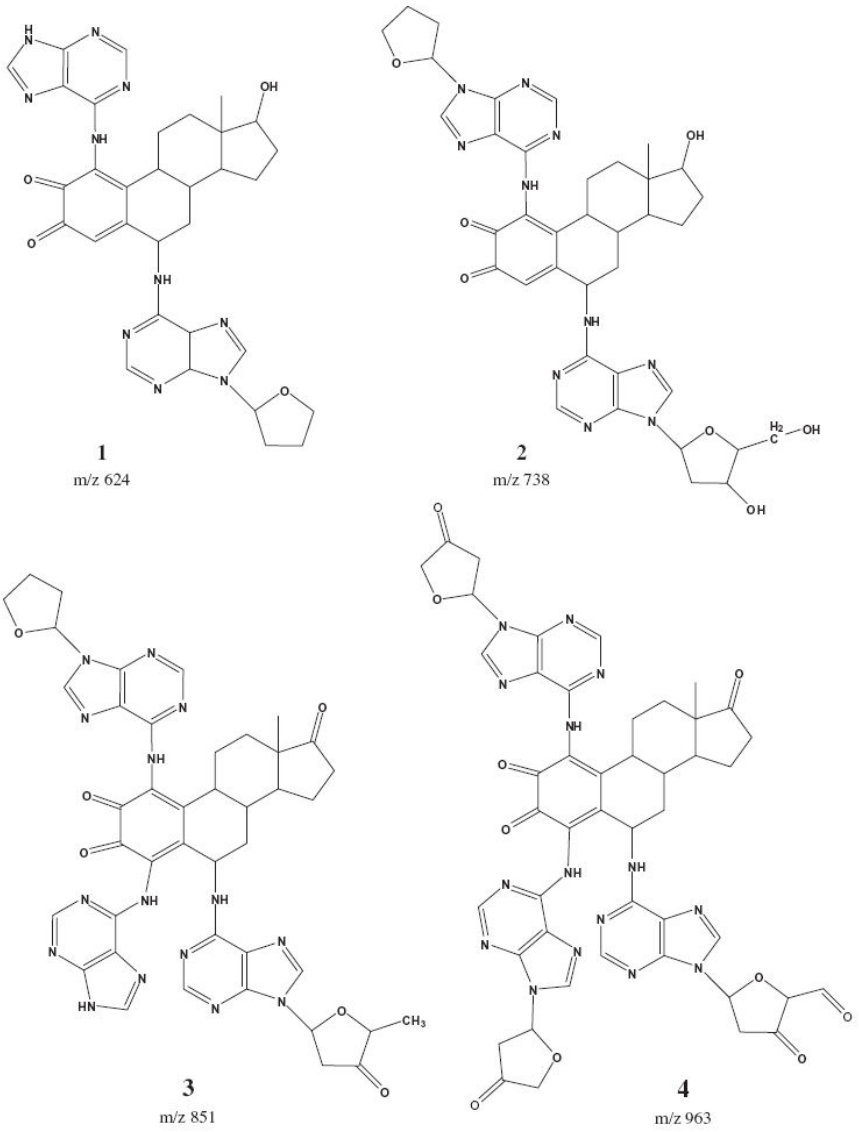


Fig. 7. Mass spectra (MS, above, and MS^2, below) for component **2**, detected in the LC–MS/MS analysis of schistosoma-infected human serum. MS and MS^2 spectra for components **1**, **3** and **4** are given on Supporting Information.

Furthermore, the proposed molecular structure of these new estrogenic molecules explains the cellular effects of Sh demonstrated in this paper. These molecules show a putative binding affinity with ER and they probably are able to antagonize estradiol activity and down-regulate ER.

Finally, to our knowledge there are no studies in the literature reporting the presence of genes involved in estrogen pathways in *S. haematobium*. As the genome sequences of the closely related African schistosome *Schistosoma mansoni* (Berriman et al., 2009) and the more distantly related Asian species *Schistosoma japonicum* (The Schistosoma japonicum Genome Sequencing and Functional Analysis Consortium, 2009) are now available we can expect that schistosomes will express all the genes necessary to synthesize estrogenic molecules. In this regard, it is noteworthy that the genomic information suggests the presence of an integral hypothalamic–pituitary–thyroid in *S. japonicum*. It was also confirmed that *S. japonicum* has receptors for steroid hormones such as progestin, progesterone and estrogen. In addition it possesses intricate

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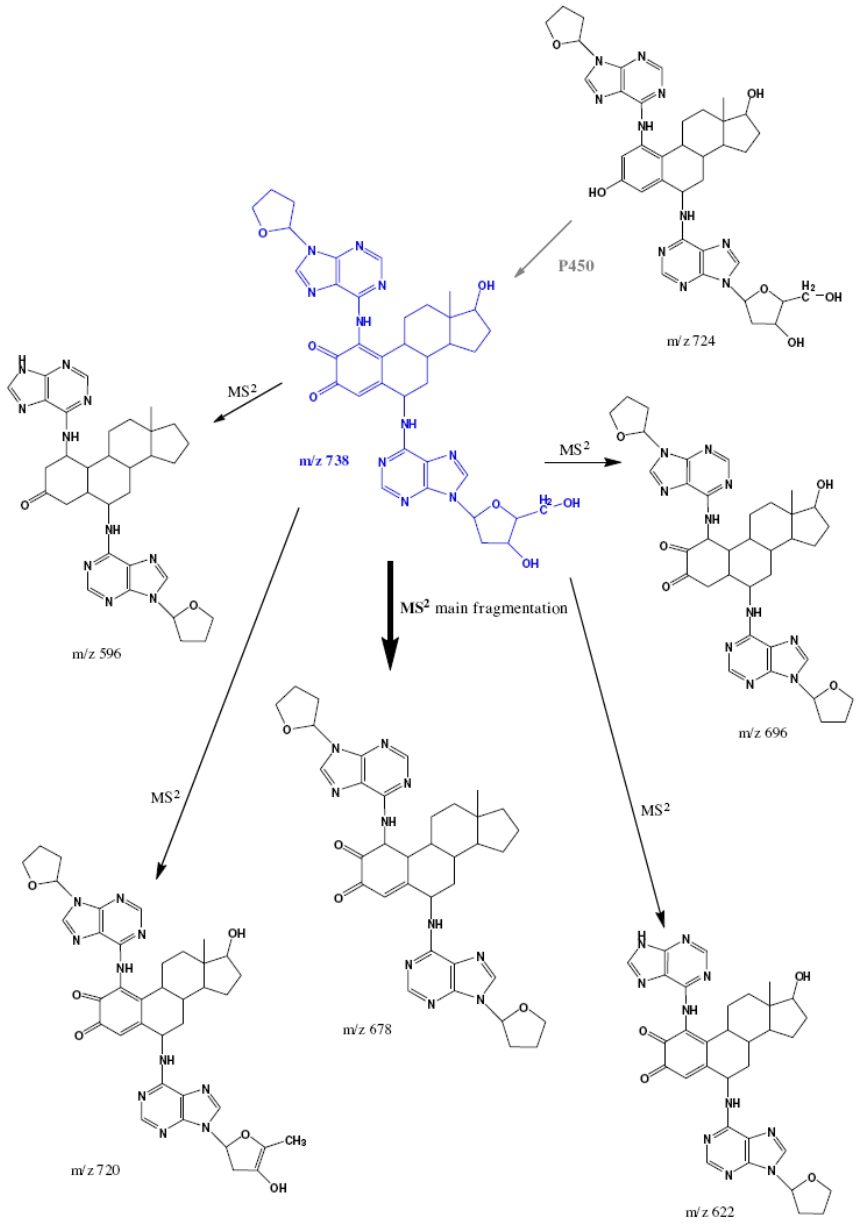


Scheme 1. Components 1, 2, 3 and 4.

pathways for processing steroid hormones to form other sex hormones. For example, there are putative enzymes present that could convert the female hormones progesterone and pregnenolone to estriol, oestrone, androsterone and testosterone. Hence, schistosomes might use these pathways during their parasitic existence (The Schistosoma japonicum Genome Sequencing and Functional Analysis Consortium, 2009).

The observations described in this study assist in defining the biochemical basis of the selective interactions between Sh and the ER, and they provide insights into the molecular mechanisms by which Sh acts to modulate the activity of this steroid hormone receptor, possible through the new estrogenic molecules identified in the crude parasite extracts. Additional studies addressing the regulation of expression of these new estrogenic molecules of *S.*

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Scheme 2. Proposed MS^2 fragmentation pathway for component 2.

haematobium and their antiestrogenic activity will be important in further understanding how this parasite infection influences ER transcription, as well as the benefit this ER repression may provide to the parasite.

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Scheme 3. Hydroxylation of both C-2 and C-3 on a steroid ring and further oxidation into an estradiol-2,3-quinone.

5. Uncited references

Botelho et al. (2009b,c).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2010.06.012.

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PAPER VIII

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Schistosoma haematobium and bladder cancer

What lies beneath?

Monica Catarina Botelho,[1,2,*] Jose Carlos Machado[2,3] and Jose Manuel Correia da Costa[1]

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Schistosoma haematobium **is a parasitic flatworm that infects millions of people, mostly in the developing world, and is associated with high incidence of bladder cancer although why is not clear. But our group was able to define the mechanistic relationship for the first time between infection of *S. haematobium* and cancer. We used in vitro models to demonstrate the presence of informative carcinogenesis-associated phenotypes in CHO cells exposed to Sh total antigen, in which we showed increased cell proliferation, decreased apoptosis, upregulation of the anti-apoptotic molecule Bcl-2, downregulation of the tumor suppressor protein p27, and increased cell migration and invasion.**

We further discuss the molecular and cellular events that might be responsible for schistosomiasis-related bladder cancer.

Key words: *Schistosoma haematobium*, squamous cell carcinoma of the bladder, CHO cells, carcinogenesis-associated phenotypes, animal models, kras mutations, estrogenic DNA adducts

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Addenda to: Botelho M, Ferreira AC, Oliveira MJ, Domingues A, Machado JC, da Costa JMC. *Schistosoma haematobium* total antigen induces increased proliferation, migration and invasion, and decreases apoptosis of normal epithelial cells. Int J Parasitol 2009; 39:1083–91; PMID: 19285502; DOI: 10.1016/j.ijpara.2009.02.016.

Schistosomiasis, also known as snail fever—since part of the parasite life cycle occurs in these animals—is a potential fatal disease that, according to the World Health Organization, infects 200 million people and is endemic in as much as 76 tropical developing countries. The disease, spread through contaminated waters, is only second to malaria in rates of infection and public health impact throughout the developing world, but has been one of many neglected tropical diseases until very recently, when globalization and its associated intense migration flux has brought it into the light. And infection by *S. haematobium*—a member of this family—is particularly relevant due to its association to bladder cancer. In fact, in some of the regions where *S. haematobium* is endemic, bladder cancer is the most common cancer in men and the second in women, just behind breast cancer, accounting for as much as 30% of all cancer cases.[1]

In an attempt to understand this worrying link between infection and cancer our group exposed cells growing in laboratory to extracts of *S. haematobium* looking for changes, particularly in those traits associated with cancerous processes. In fact, cancer cells can be defined by rapid uncontrolled division, high resistance to death and—in the late stages of the disease—an abnormal capability to migrate through tissues (normal cells, except for a few exceptions, are not capable of move out of their "home" tissue/organ). We found that, in fact, cells exposed to *S. haematobium* cells divided faster and more than those not exposed to the parasite, and also died much less. When searching for molecular clues to explain such alterations it was found that the altered cells presented increased levels of bcl2—a protein involved in cellular death and linked to cancer—while the tumor suppressor protein p27 was reduced. These cells were also more mobile than controls, a crucial characteristic for metastasis formation. These results revealed *S. haematobium* is able to induce the formation of cancer-like cells.[2]

Next, in a second study we injected *S. haematobium*—exposed cells into mice with no immune system and, remarkably, this led to tumors very similar to those found in bladder cancer. Crucially, animals injected with non-exposed cells showed no growths (**Fig. 1**).[3]

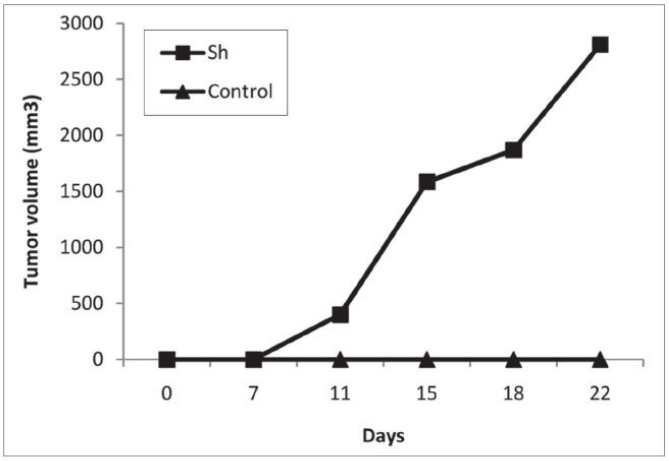


Figure 1. Analysis of the tumour volume induced by control CHO and Sh treated CHO in nude mice. Tumour volumes were measured at the indicated days after injection. Each point represents the mean of five individual determinations.

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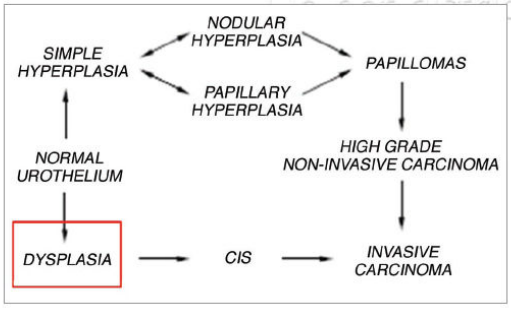


Figure 2. Pathogenic pathways of rat and mice urinary bladder carcinogenesis (reviewed in ref. 3).

We have further used a CD-1 mice model to show that *Schistosoma haematobium* total antigen (Sh) has a carcinogenic ability. Intravesically administration of Sh gave rise to a high incidence of urothelial dysplasia (**Fig. 2**).[4]

The urothelium of the animals exposed to Sh described in this paper presented similar phenotypes. The urothelium of these animals presented dysplasia (LGIUN—Low Grade Intra-Urothelial Neoplasia), a non-invasive malignant flat lesion, in 70% of the tested subjects after 40 weeks of treatment (**Table 1, Fig. 3**). Our results suggest that Sh induce the malignization of the urothelium.[5]

Although squamous cell carcinoma of the urinary bladder has been associated with *Schistosoma haematobium* infection in many parts of Africa, the cellular and molecular mechanisms linking *S. haematobium* infection with cancer formation are not yet defined. Carcinoma of the bladder frequently harbours gene mutations that constitutively activate the receptor tyrosine kinase-Ras pathway.[6] The ras-signaling pathway has attracted considerable attention as a target for anticancer therapy because of its important role in carcinogenesis. The Ras gene product is a monomeric membrane-localized G protein of 21 kd that functions as a molecular switch linking receptor and nonreceptor tyrosine kinase activation to downstream cytoplasmic or nuclear events. Each mammalian cell contains at least three distinct ras proto-oncogenes encoding closely related, but distinct proteins, Kras, Hras and Nras Activating mutations in these Ras proteins result in constitutive signaling, thereby stimulating cell proliferation and inhibiting apoptosis. Oncogenic mutations in the ras gene are present in approximately 30% of all human cancers.[7] In our latest study we used the dysplastic bladders induced by Sh and screened them by sequencing

Table 1. Histopathological findings in the Sh instilled bladders and controls

Group	n	W	N	U	I	L	M	D
Group 1 (Sh treated)	10	20	7/10 (70%)	5/10 (50%)	9/10 (90%)	9/10 (90%)	0/10 (0%)	3/10 (30%)
Group 2 (Control)	10	20	10/10 (100%)	9/10 (90%)	2/10 (20%)	2/10 (20%)	0/10 (0%)	0/10 (0%)
Group 3 (Sh treated)	10	40	0/10 (0%)	0/10 (0%)	10/10 (100%)	9/10 (90%)	4/10 (40%)	7/10* (70%)
Group 4 (Control)	10	40	10/10 (100%)	10/10 (100%)	3/10 (30%)	3/10 (30%)	0/10 (0%)	0/10 (0%)

n, number of animals; W, weeks between treatment and sacrifice; N, normal urothelium; U, presence of umbrella cells; I, inflammatory infiltrate; L, infiltration of lymphocyte; M, infiltration of mastocytes; D, dysplasia; *(p = 0.001, Group 3 Vs. Group 4).

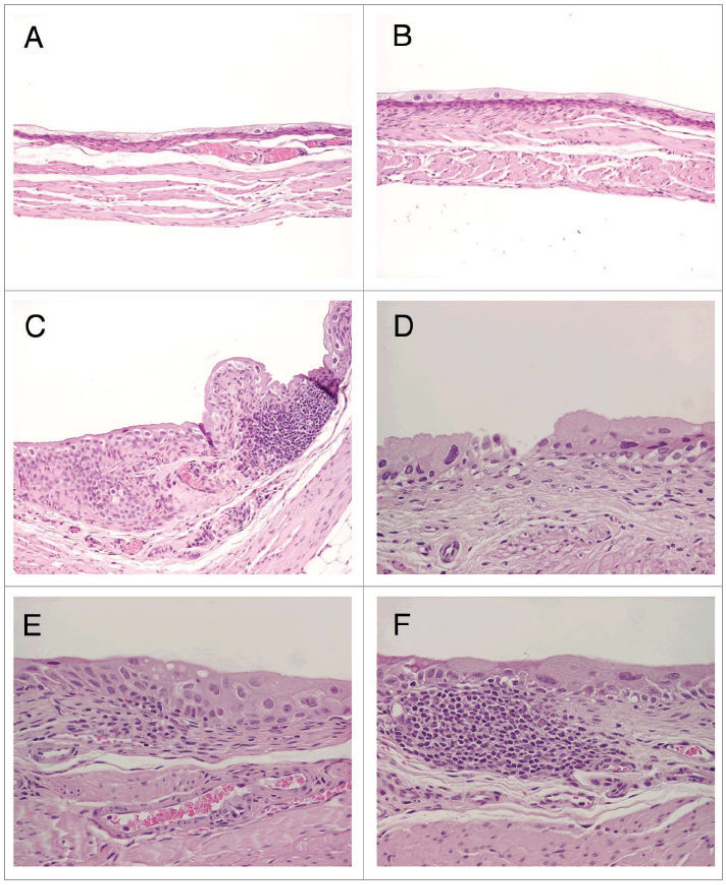


Figure 3. (A and B) control, (C) urothelium displaying an inflammatory nodule and a lesion with characteristics of nodular hyperplasia, (D) denudation and dysplasia, (E) inflammatory infiltrate and dysplasia, (F) inflammatory nodule and dysplasia.

for mutations in the Kras codon hotspots (12 and 13) gene. Two (out of 10) of the bladders with dysplasia presented a KRAS mutation in codon 12 of exon 2 (**Fig.** 4). We concluded from these results that the parasite extract of *S. haematobium* has carcinogenic ability possibly through oncogenic mutation of KRAS gene.

Further investigations using *Schistosoma haematobium* antigens and the identification of the carcinogenic component(s) may be useful to understand oncogenesis associated with this parasite. We have attempted to identify such carcinogenic compounds and they are probably quinone forms generated by metabolic activation of catechol estrogens. In a first report, high levels of oestradiol but not LH, and Testosterone were detected in sera from urinary schistosomiasis patients.[8] This evidence was unexpected. Estradiol is a steroid hormone secreted principally by the ovarian follicles in vertebrate animals. How could this be possible? The hormone levels in sera patients neither could nor be attributed to thyroidal-hypothalamic axe. How to explain this non sense biological evidence? The production of an estradiol-related molecule by Schistosoma was hypothesized. Finally, we show that this molecule related to estradiol is present in schistosome worm extracts. In a first attempt, the detection method ELISA specific for estradiol, revealed the expression of this estradiol-related molecule in schistosome worm extracts, but not in *Fasciola hepatica* worm extracts. We were able to characterize 4 estrogenic molecules. These molecules probably mediated an interaction between the parasites and their hosts, because their presence was detected in both organisms.

The quinone forms generated by metabolic activation of catechol estrogens are highly electrophilic species. Some authors

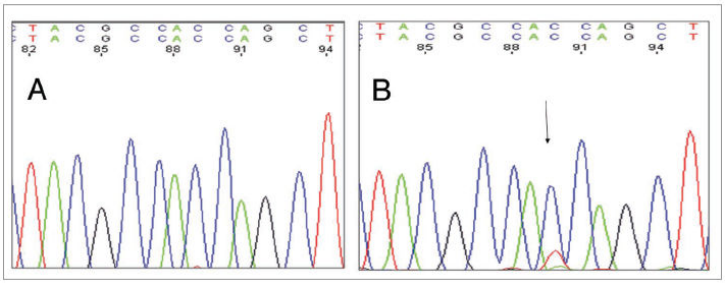


Figure 4. *KRAS* exon 2 sequencing results. (A) control, (B) dysplastic bladder with a mutation at codon 12 (resulting in a Gly12Asp mutation).

reported that these species are able to covalently bind DNA bases, forming stable adducts.[9-12] These adducts can be considered as estrogen-bound metabolites generated in vivo that are produced by nucleophilic attack of the DNA base on different positions of the steroid ring.[10-13] Catechol estrogen-3,4-quinone adducts of DNA have been proposed as endogenous initiators of breast, prostate and other human cancers.[14]

The carcinogenic effect of this estrogen adduct-mediated pathway of the estrogenic molecules present in *S. haematobium* extracts could explain the link between this parasite and squamous cell carcinoma of the bladder. More studies are ongoing aiming a better comprehension of these biological interactions and their carcinogenic effect.

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PAPER IX

Research Paper

Inactivation of Estrogen Receptor by *Schistosoma haematobium* total antigen in bladder urothelial cells.

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Running Title: *Schistosoma haematobium* is an antagonist of estradiol and represses ER.

Abstract

We recently reported the expression of an estradiol-like molecule by a trematode parasite *Schistosoma haematobium*. We further established that this estradiol-like molecule is an antagonist of estradiol, repressing the transcriptional activity of the Estrogen Receptor (ER) in estrogen responsive MCF7 cells and also that *S. haematobium* total antigen (Sh) contains estrogenic molecules detected by mass spectrometry.

In the present work we used HCV29 cells, a cell line of normal urothelial cells, and an in vivo model to evaluate the expression of ER in the bladders of Sh instilled animals. We show that, similarly to MCF7 cells, Sh is able to repress the transcriptional activity of ER in HCV29 cells and also in the bladders of Sh-treated mice.

The activity of *S. haematobium* estrogens and its repressive role in ER could have implications in the carcinogenic process of the bladders in *S. haematobium* infection.

Keywords: *Schistosoma haematobium*, estradiol, estrogen receptor, MCF-7 cells, CHO cells.

1. Introduction

Schistosomiasis is the first in prevalence among all water-borne infectious diseases and one of the main occupational risks encountered in the rural endemic areas of the world (Badawi AF, 1996). It is a common parasitic disease that ranks second only to malaria as a global cause of morbidity and mortality. It occurs in 76 tropical and subtropical countries and affects about 10% of the world's population. The prevalence of this infection remains high despite the availability of efficacious antischistosomal drugs. Individuals become infected with the parasite at an early age after exposure to the free-swimming larval stage (termed cercariae) in contaminated water (Rosin MP, 1994). Despite affecting 200 million people, schistosomiasis remains a neglected disease. There are two main reasons for this: the availability of an effective and cheap drug, praziquantel, has contributed to the wrong perception that chemotherapy would represent the final solution for the control of schistosomiasis, and the disability adjusted life years as a measure of the impact of disease has been under evaluated because of rebound morbidity following chemotherapeutic intervention (Capron et al, 2005).

Estrogen receptors are known to mediate important physiologic functions, including reproduction, metabolism, maintenance of bone density, and growth of estrogen-responsive tumors, including breast and endometrial cancers. They are members of a superfamily of nuclear receptors that are able to transducer extracellular signals into transcriptional response. There are 2 nuclear receptors, ER alpha and ER beta, which are known to mediate the physiologic response to estrogen. They share several functionally conserved structural domains such as the regions responsible for DNA binding, dimerization, ligand binding and ligand-dependent transactivation of gene expression (Shen SS et al, 2006). ER alpha and beta have different transcriptional activities in certain ligand, cell-type, and promoter contexts and also exhibit distinct tissue- and cell-type specific expression patterns. ER alpha is found predominantly in the normal pituitary, breast, uterus, vagina, testis, liver, and kidney, whereas ER beta is mainly expressed in thyroid, ovary, prostate, skin, bladder, lungs, gastrointestinal tract, bone and cartilage (Shen SS et al, 2006).

We recently reported the expression of an estradiol-like molecule by a trematode parasite *Schistosoma haematobium* (Botelho MC et al, 2009). We further established that this estradiol-like molecule is an antagonist of estradiol, repressing the transcriptional activity of the Estrogen Receptor (ER) in estrogen responsive MCF7 cells and also that *S. haematobium* total antigen (Sh) contains estrogenic molecules detected by mass spectrometry (Botelho MC et al, 2010).

In the present work we used HCV29 cells, a cell line of normal urothelial cells, and an in vivo model to evaluate the expression of ER in the bladders of Sh instilled animals. We show that, similarly to MCF7 cells, Sh is able to repress the transcriptional activity of ER in HCV29 cells and also in the bladders of Sh-treated mice.

The activity of *S. haematobium* estrogens and its repressive role in ER could have implications in the carcinogenic process of the bladders in *S. haematobium* infection.

2. Materials and Methods

Animals

Eight-week-old female golden hamsters (LVG/SYR) and CD-1 mice were provided by Charles River (Barcelona, Spain). Animals spent one week being acclimated under routine laboratory conditions before starting the experiments. They did not receive any treatment prior to the study. Hamsters were kept in separated cages and mice were kept in 6 litter mates cage. 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 ± 2ºC) 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

Urine was collected from S. haematobium-infected individuals. The individuals were linving in Angola, an endemic area for schistosomiasis. Following instruction in midstream urine collection, urine samples were then collected from each individual. S. haematobium infection was detected by microscopically observation of the eggs in the sediment of centrifuged urine. Informed consent from patients was obtained. The eggs were hatched and with the resulting miracidia, snails from susceptible species were infected.

Golden hamsters were experimentally infected with 100 cercariae by tail immersion. The control animals consisted of littermates. The cercariae were obtained by shedding of snails infected with miracidia.

S. haematobium total antigen (Sh) production

S. haematobium adult worms (Angolan strain) were collected by perfusion of the hepatic portal system of golden hamsters at seven weeks after infection with 100 cercarie. The worms were suspended in PBS and then sonicated. The protein extract was then ultracentrifuged and the protein concentration was estimated using a micro BCA protein assay reagent kit.

Experimental design

Forty mice were randomly divided into six experimental groups. Groups 1 and 3 (n=10) were Sh-instilled animals. Groups 2 (n=5) and 4 (n=5) were control animals with their bladders instilled with saline only. Groups 1 and 2 were sacrificed 19 weeks after treatment; group 3 and 4 were sacrificed 32 weeks after treatment. For the duration of the study, the mice's state of health was monitored daily.

For intravesical instillation of the parasite total antigen, 50 μg/ml of Sh was introduced directly into the urinary bladders of female mice via the urethra according to the procedure previously described [11]. Briefly, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). The bladder was catheterized via the urethra with a 24 gauge plastic intravenous cannula and the bladder mucosa was then traumatized to remove the umbrella cells of the urothelium by instilling 0.1 ml of 0.1N HCl solution for 15 seconds, neutralized with 0.1 ml of 0.1N KOH and flushed with sterile saline.

A total of 150 μl of Sh with a concentration of 50 μg/ml was instilled via the cannula. The dwell time after instillation was approximately 1 hour. The mice were turned 90 degrees every 15 minutes to facilitate whole bladder exposure to the Sh intravesically instilled. One hour after the instillation, micturition of the mice was induced by light abdominal massage so that the duration of exposure was constant. Body temperature was maintained with a homoeothermic bandage. All the experiments described herein were carried out under aseptic conditions and performed once during this study.

All animals were sacrificed by intraperitoneal administration of sodium pentobarbital (130 mg/Kg). Complete necropsies were carefully conducted. All organs were examined macroscopically for any changes. The urinary bladders were inflated in situ by injection of 10% phosphate buffered formalin (150μl), ligated around the neck to maintain proper distension and then immersed in the same solution for 12 hours. After fixation, the formalin was removed; the urinary bladder was cut into two strips, and routinely processed. Tissue sections (2 μm) from urinary bladder, spleen, liver and lung were stained with haematoxylin and eosin (HE) to characterize changes and inflammatory infiltrate.

Chemicals and materials

HCV29 cells were cultured in RPMI 1640 medium phenol red free (Sigma Aldrich, Portugal), with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, purchased in Invitrogen Life Technologies (Paisley, Scotland, UK). Estradiol (E2) was obtained from Sigma Aldrich (Portugal) and the antiestrogen ICI 182,780 (ICI) was kindly provided by AstraZeneca (Portugal), both chemicals dissolved in 100% ethanol and added to cell culture medium.

Cell culture

HCV29 cells were maintained at 37º C in a 5% CO2 humidified atmosphere. Cells were passaged every 5 days. Before treatments cells were serum-starved for 16 h. The cells were treated with Sh antigen for 24 h in serum-free conditions.

Lactoferrin assay

MCF-7 cells were cultured in 0.3 ml RPMI medium in 24-well flat-bottomed plates. Cultures were seeded at $5x10^4$ cells / well and allowed to attach overnight. Cells were then treated with 50 μg/ml of Sh for 24h. Lactoferrin in the supernatant of MCF-7 cells was determined using ELISA with an antibody specific for lactoferrin (Hycult, Netherlands) according to the manufacturer's instructions.

Isolation of RNA

At defined time intervals MCF-7 cultured cells were harvested in Tripure reagent (Roche, Portugal). Total RNA was isolated from the cell material according to the manufacture instructions. RNA recovery and quality were checked by measuring the optical density ratio (260/280 nm) and its quantification was determined also by optical density (Nanodrop).

Real-Time PCR

Real-Time PCR was performed in HCV29 cultures. For this purpose cDNA was obtained using the Promega cDNA kit. For each sample, 1.0 µg of RNA was reverse transcribed in a reaction volume of 20 µl in the presence of 10 mM deoxynucleotide triphosphate and 2 µl RT-PCR enzyme mix. Taqman GeneExpression assays (Applied biosystems) were used for gene-specific primers and probes for ER alpha (ESR1, PE Applied Biosystems, USA) and ER beta (ESR2, PE Applied Biosystems, USA). Both target gene transcripts were normalized to the endogenous control GAPDH mRNA (PE Applied Biosystems, USA) content and to the control sample, through the $-2^{\Delta\Delta Ct}$ semi-quantitative method. Quantifications were performed in triplicate in a 7500 Real-Time PCR System (PE Applied Biosystems, USA).

Estrogen receptor immunohistochemistry

Immunohistochemistry was performed in 5 µm sections attached to silane coated slides that were dried for 48 to 72 hours at 37 ºC before use. After dewaxing in xylene and rehydration through graded alcohol to water, antigens were retrieved using microwaving at 850 W in 10nM citrate buffer, pH 6,0, for 10 minutes and allowed to cool. After a brief wash in distilled water endogenous peroxidise activity was quenched by incubation with 3% hydrogen peroxide for 10 minutes. Nonspecific binding sites were blocked with 10% normal rabbit serum (Dako, Glostrup, Denmark) diluted in phosphate buffered saline (PBS). Sections were incubated with primary mouse monoclonal antibody against ER (Zymed, San Francisco, CA, U.S.A.) in a humidified container for 18 hours at 4ºC. After washing with the appropriate PBS for 20 minutes the sections were incubated with biotinylated rabbit anti-mouse Igs (Dako; 1:400) for 30 minutes at room temperature. After another wash with buffer for 20 minutes specimens were incubated with Vectastain ABC Elite avidin-biotin complex linked horseradish peroxidise reagent for 30 minutes at room temperature in a humidified chamber. After a further wash with buffer for 30 minutes immunoreactivity was visualized with exposure to 3,3'-diaminobenzidine substrate

(Vector Laboratories, Peterborough, United Kingdom) for 5 minutes. Sections were briefly counterstained with Mayer's hamatoxylin (Sigma).

Immunohistochemistry analysis

The distribution of positively stained cells in the urothelium, urethral epithelium and smooth muscle cells was determined as a percentage of positive cell nuclei counted. The intensity of the reaction was recorded as follows: negative (-), weak (1+), moderate (2+), and strong (3+).

Statistical analysis

For *in vitro* experiments data were expressed as mean ± SD. *t* test was used to assess the statistical significance of differences. $P < 0.05$ was considered statistically significant.

For animal experiments statistical analysis was performed using Microsoft Excel Analysis ToolPak for Windows. The differences in expression of Estrogen Receptor between groups were assessed by use of the chi-square test. A $p<0.05$ was accepted as statistically significant.

3. Results

S. haematobium total antigen reduces the production of lactoferrin in the supernatant of HCV29 cells

Lactoferrin is a target gene of Estrogen Receptor and can be a useful marker of estrogen action on estrogen responsive cells. Therefore HCV29 cells were seeded on 96 well plates, starved ON, treated with 50 μg/ml of Sh for 24 h, cultured for 24 and 48 hours, and the supernatants analyzed by ELISA for lactoferrin (Figure 1). Bars show that Sh-treated cells produced less lactoferrin than control cells. In addition, incubation with ICI or Sh+ICI also led to identical decrease in lactoferin secretion (*p< 0.05 vs Controls).These results suggest that Sh treatment represses Estrogen Receptor activity in HCV29 cells.

***Schistosoma haematobium total antigen down-regulates ER alpha and ER beta* in HCV29 cells**

To confirm the involvement of estrogen receptor, expression of *ESR1* and *ESR2* genes were analyzed in HCV29 cells after estradiol, ICI and Sh treatment. Incubation with estrogen clearly increased both ER alpha and ER beta expression (Figure 2). Furthermore, both ER isoforms were downregulated by Sh treatment in this cell culture, either alone or in the presence of ICI in comparison to control (Figure 2). Interestingly, Sh incubation extensively reduced *ESR1* and *ESR2* expression, even in the presence of estrogen (*p< 0,05 Sh+E2 vs. E2).

***Schistosoma haematobium total antigen down-regulates ER* expression in the bladders of CD1 mice**

A total of 30 bladders, 10 controls and 20 treated animals, were analysed for the ER expression. The urothelium was always negative for ER protein expression in all animals of the 4 groups. The controls of Group 2, sacrificed after 20 weeks, showed positive staining in the urethral epithelium and in the smooth muscle cells (SMC) in 80% (4 out of 5) of the bladders (Table 1, Figures 3A, 3B, 3C and 3D). The treated bladders were negative for ER expression in the urethral epithelium (Table 1, Figure 3E) and SMC (Table 1, Figure 3F) in 60% (6 out of 10) of the bladders of mice belonging to Group 1 which were sacrificed after 20 weeks. The bladders of the animals in this group that were considered positive for ER expression (40%) exhibited a week immunostaining. The treated animals of Group 3, sacrificed after 40 weeks, were ER negative for urethral epithelium and SMC in 50% (5/10) of the bladders. The other half showed a pattern of staining similar to the positive bladders of Group 1 (Table 1). Group 4, controls sacrificed after 40 weeks, showed positive ER expression in the urethral epithelium and in the smooth muscle cells (SMC) in 60% (3 out of 5) of the bladders.

4. Discussion

This study addresses the suppression of ER transcriptional activity in urothelial cells and ER expression in the bladders of mice in response to Schistosoma haematobium. Data presented here add new observations to this area by demonstrating that 1) S. haematobium extracts reduce the production of lactoferrin by urothelial cells, 2) urothelial cells produce both transcripts of ER and they are downregulated by S. haematobium extracts, and 3) the bladders of mice instilled with S. haematobium extracts exhibit downregulation of ER in urethral epithelium and smooth muscle cells in contrast with controls.

We have previously shown that this parasite extracts had an antagonistic effect of estradiol *in vitro*. We observed decreased production of lactoferrin, an estradiol target gene, in the supernatants of the Sh-treated estrogen responsive cell line MCF-7 and down-regulation of both ER alpha and beta in these cells (Botelho M et al, 2010). We now demonstrate that this effect also occurs in urothelial cells and in the bladders of Sh-treated mice. The bladders of mice exhibit downregulation of ER in urethral epithelium and smooth muscle cells in contrast with controls, but we didn't find expression of ER in the bladder urothelium of controls and Sh-treated mice. In accordance, ER was found to localize to the urethral epithelium but has been reported to be absent from transitional epithelium (Tincello DG et al, 2009).

The present work addresses the regulation of *S. haematobium* antiestrogenic activity as an important mechanism used by the parasite to influence ER transcription. The benefit that this ER repression may provide to the parasite could be explained by the following hypothesis: estradiol treatment has been shown to increase smooth muscle density in the bladder of female rats (Xin Y et al, 2009). In our study we observed downregulation of ER expression in the smooth muscle of Sh-treated bladders. During infection with *S. haematobium* the parasite eggs must cross the bladder mucosa to reach the lumen of the bladder to be excreted, in order to survive and continue its life cycle. A thinner smooth muscle layer in the bladder mucosa of infected individuals could possibly increase the number of eggs released.

On another level, the repression of ER during *S. haematobium* infection could be a collateral damage of the infection. Nevertheless ER repression could have implications in the long term sequella of bladder cancer associated with this infection. We have previously identified by Mass Spectometry in S. haematobium extracts and in the serum of infected individuals, four new estrogenic molecules that are capable, according to their molecular structure, to bind ER and exert the repression effect observed (Botelho M et al, 2010). The molecules detected on the LC–MS analysis of both the schistosoma extract and the plasma of a schistosoma-infected human were formed by reactions of estrogen-quinones with DNA (Scheme 1). The quinone forms generated by metabolic activation of catechol estrogens are highly electrophilic species. Some authors reported that these species are able to covalently bind DNA bases, forming stable adducts (Stack DE et al, 1996; Cavalieri EL et al, 1997; Akanni A et al, 1997a; Akanni et al, 1997b). These adducts can be considered as estrogen-bound metabolites generated in vivo that are produced by nucleophilic attack of the DNA base on different positions of the steroid ring (Cavalieri EL et al, 1997; Akanni A et al, 1997a; Akanni et al, 1997b; Farmer PB et al, 1995). Catechol estrogen-3,4-quinone adducts of DNA have been proposed as endogenous initiators of breast, prostate and other human cancers (Zahid M et al, 2006). In such cases, oxidation of the catechol estrogen metabolites 4- and 3-hydroxyestradiol would lead to formation of, respectively, estradiol-3,4-quinone and estradiol-2,3-quinone, whose reactions with DNA have shown to be much more reactive and correlated with the carcinogenicity and mutagenicity of their precursors Zahid M et al, 2006. The apparently higher reactivity of estradiol-3,4-quinone with DNA may be possibly due to its longer half-life, providing extended reaction time with DNA (Iverson SL et al, 1996). Although evidence from cell-culture and animal studies support the estrogen adduct-mediated pathway for the referred cancers, data from humans are scarce. Some authors have reported 4-OH-E1-1-N3Ade at 8.4 pmol/g of breast tissue from a woman with carcinoma (Markushin Y et al, 2003), and a nondepurinating hydroxyestradiol guanosine found upon hydrolysis of DNA from breast tissues (Embrechts J et al, 2003). Interestingly, urine levels of 4-OH-E1-1-N3Ade are low in men with prostate cancer or other urological problems (Markushin Y et al, 2006), whereas a study involving 76 women showed high urine levels of that estrogen adduct for the women at high risk or with breast cancer, as compared to controls (Gaikwad NW et al, 2008). Aerden and co-workers (1998)

developed an LC-MS/MS method for the assessment of the reactivity of estradiol-2,3-quinone towards deoxyribonucleosides in crude reaction mixtures and proved that LC-ESI-MSn is a very powerful analytical tool for the detection of DNA-estrogen adducts generated in vivo. Also, Zhang et al (2008) have recently described an extraction procedure and a LC-MS/MS assay to detect estrone-metabolite-modified adenine in 100-200 mg samples of human breast tissue. The carcinogenic effect of this estrogen adduct-mediated pathway of the estrogenic molecules present in S. haematobium extracts could explain the link between this parasite and squamous cell carcinoma of the bladder. In accordance, we have previously demonstrated that normal cells treated in laboratory with S. haematobium total antigen, acquire cancer-like characteristics. In fact, these cells presented rapid uncontrolled division, high resistance to death and an abnormal capability to migrate (Botelho M et al, 2009b) and, when injected into mice with no immune system, lead to the formation of tumours. Crucially, animals injected with non-exposed cells showed no growth (Botelho M et al, 2009c). We also demonstrated that S. haematobium total antigen in CD-1 mice normal bladders after intravesical administration of the parasite antigens induced the development of urothelial dysplasia and inflammation (Botelho M et al, 2010b). The increased migration and invasion of CHO cells induced by Sh previously observed (Botelho M et al, 2009b) could be explained by the estrogens found in Sh. Corroborating this hypothesis, Maynadier and collaborators (2008) demonstrated that estradiol inhibit invasion in the ER alpha –negative MDA-MB-231 cell line transiently transfected with ER alpha, and that this inhibitory effect is reversed by antiestrogens.

In summary, this study reports the abrogation of ER expression in urothelial cells after treatment with S. haematobium extract. Both ER alpha and ER beta are downregulated in these cells by the parasite. Moreover, we also demonstrate that the instillation of S. haematobium extract in the bladders of mice downregulates the expression of ER in the urethral epithelium and in smooth muscle cells. These findings may have relevance to the development of bladder cancer associated with S. haematobium infection. Future work should assess the specific effects of the estrogenic molecules previously identified in the parasite extract.

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Legends to Figures

Figure 1: Lactoferrin production in supernatant of HCV29 cells. Cells were treated 50 μg/ml of Sh. Data represent means ±SEM from 3 independent experiments ($p<0.05$ Sh vs. Control; ICI vs. Control; and Sh+ICI vs. Control).

Figure 2: Real-Time PCR for ER alpha and ER beta in HCV29 cells. Bars correspond to fold increase of ER alpha and ER beta gene expression after normalization to endogenous control gene GAPDH and control sample. Results are representative of three independent experiments. Statistically significant differences in ER alpha and ER beta gene expression were found between Sh-treated and vehicle-treated HCV29 cells (*, $p< 0.05$ vs. control).

Figure 3: Immunohistochemistry staining for ER in the bladders of mice. A and B, urothelium of a control animal showing negative staining for ER; C, urethral epithelium of a control animal showing stained nuclei (400x); D, SMC of a control animal also with positive staining (400x); E, urethral epithelium of Sh-treated mouse with negative expression (400X); F, SMC lining the urethral epithelium of Sh-treated mouse also negative for ER expression (400x).

Scheme 1: Estrogenic molecules identified in *S. haematobium* extracts. These new estrogenic molecules were detected on the LC-MS/MS analysis and formed by reactions of estrogen-quinones with DNA.

TABLE 1

Table 1: Expression of ER in Sh-instilled bladders and control groups					
	Evaluated structures	Number of samples showing the stated estrogen-receptor immunoreactivity (%)			
Group		Negative	Weak (+)	Moderate (++)	Strong (+++)
1 (n=10)	Urothelium	10(100)		-	-
	Urethra	6(60)	4(40)	-	-[a]
	Smooth muscle	6(60)	4(40)	-	-[b]
2 (n=5)	Urothelium	5(100)	-	-	-
	Urethra	1(20)	-	3(60)	1(20)
	Smooth muscle	1(20)	-	3(60)	1(20)
3 (n=10)	Urothelium	10(100)	-	-	-
	Urethra	5(50)	5(50)	-	-[c]
	Smooth muscle	5(50)	5(50)	-	-[d]
4 (n=5)	Urothelium	5(100)	-	-	-
	Urethra	2(40)	-	2(40)	1(20)
	Smooth muscle	2(40)	-	2(40)	1(20)

[a] (p=0,01 urethra Group 1 vs. Group 2); [b] (p=0,01 smooth muscle Group 1 vs. Group 2); [c] (p=0,03 urethra Group 3 vs. Group 4); [d] (p=0,03 smooth muscle Group 3 vs. Group 4)

Figures

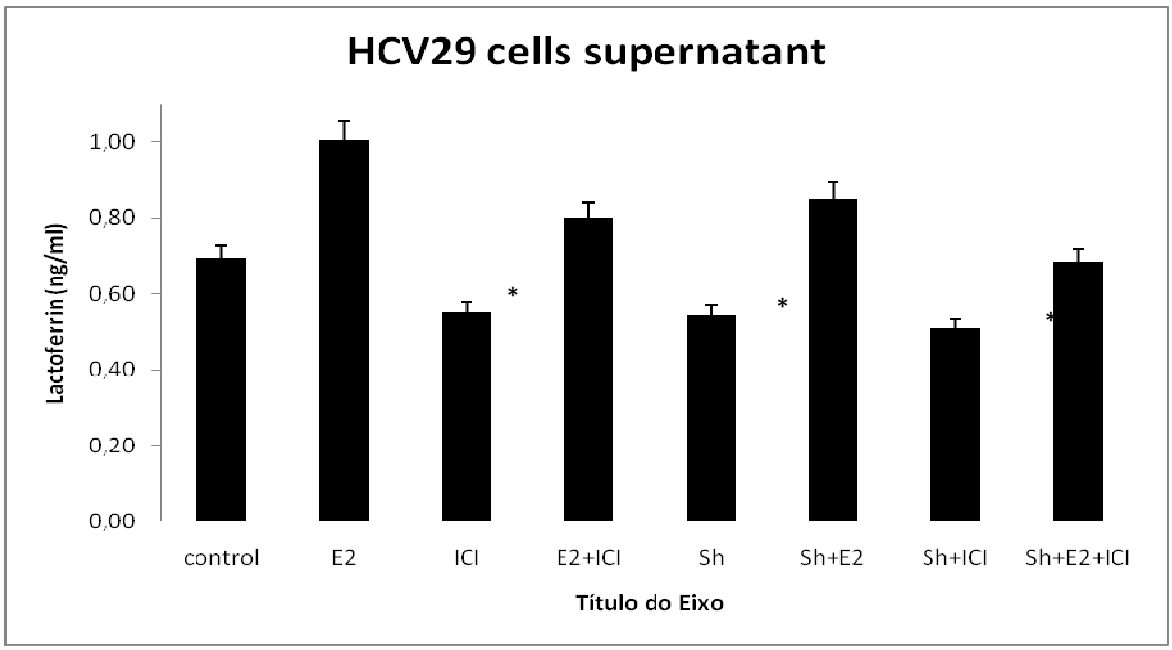


Figure 1

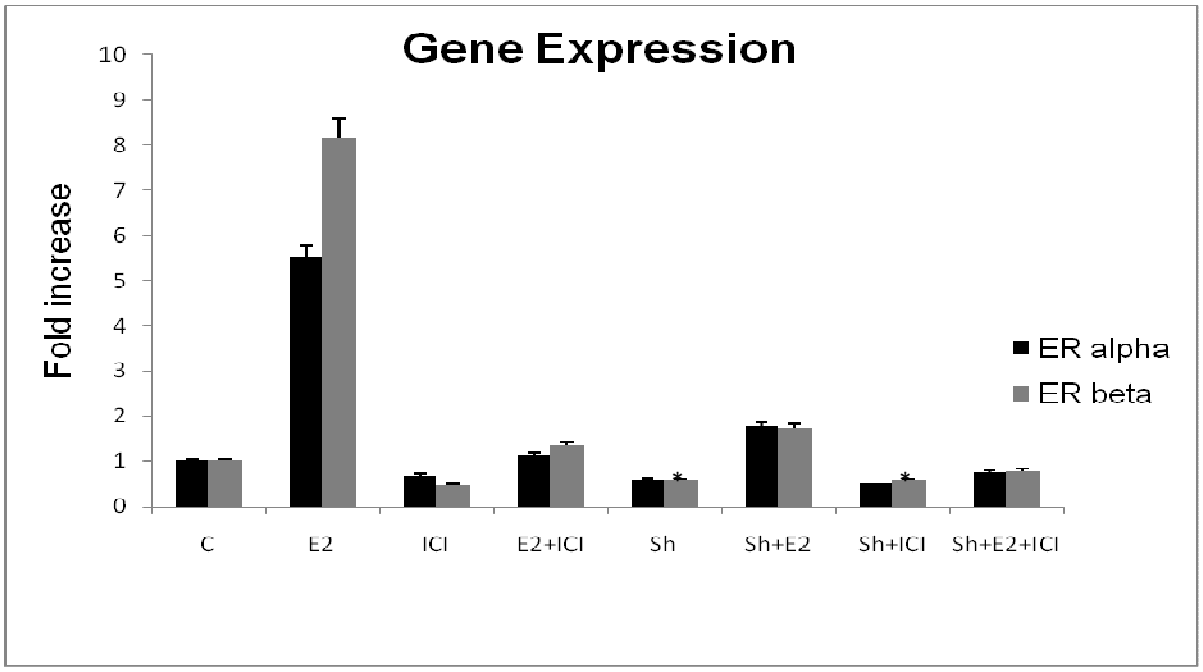


Figure 2

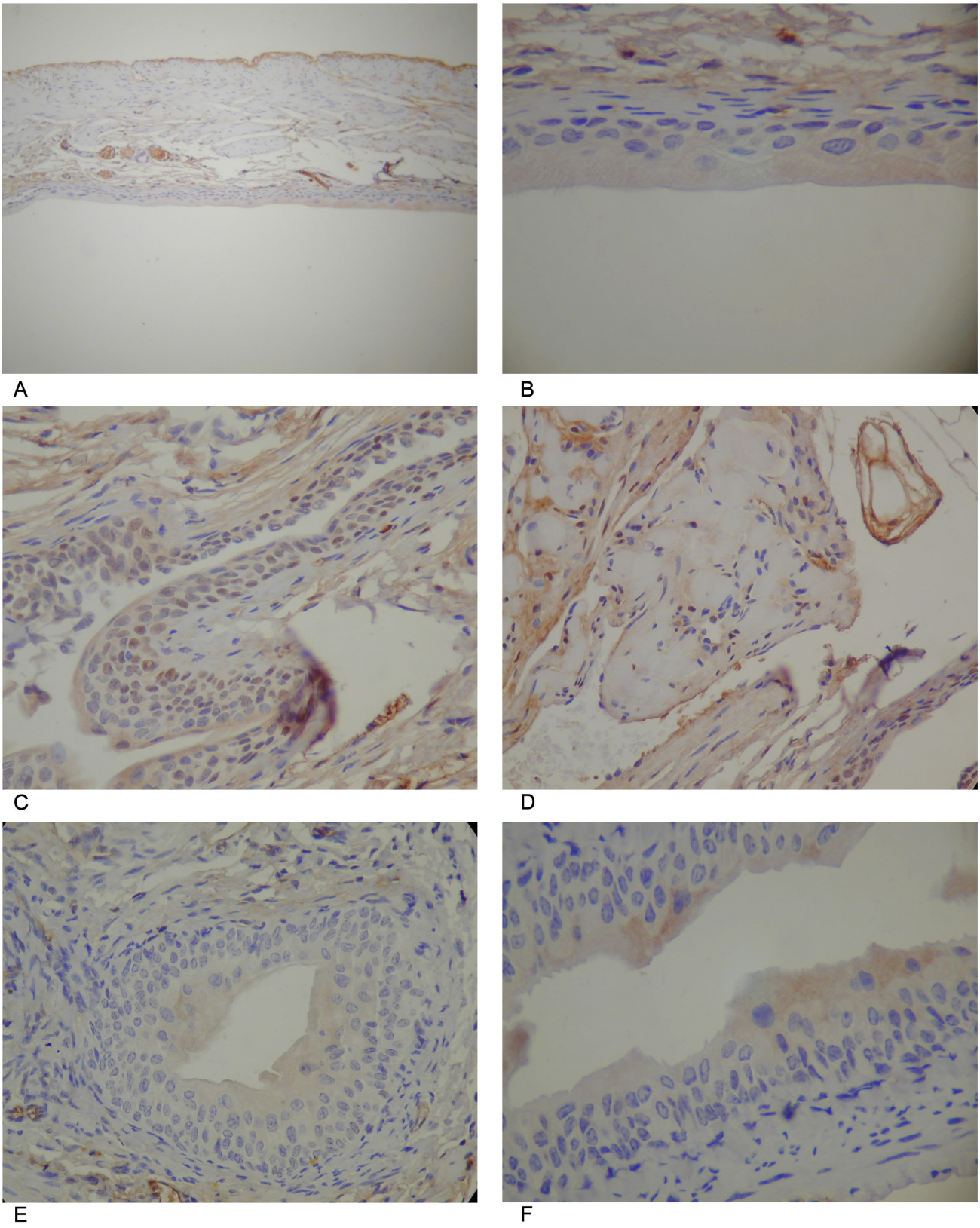
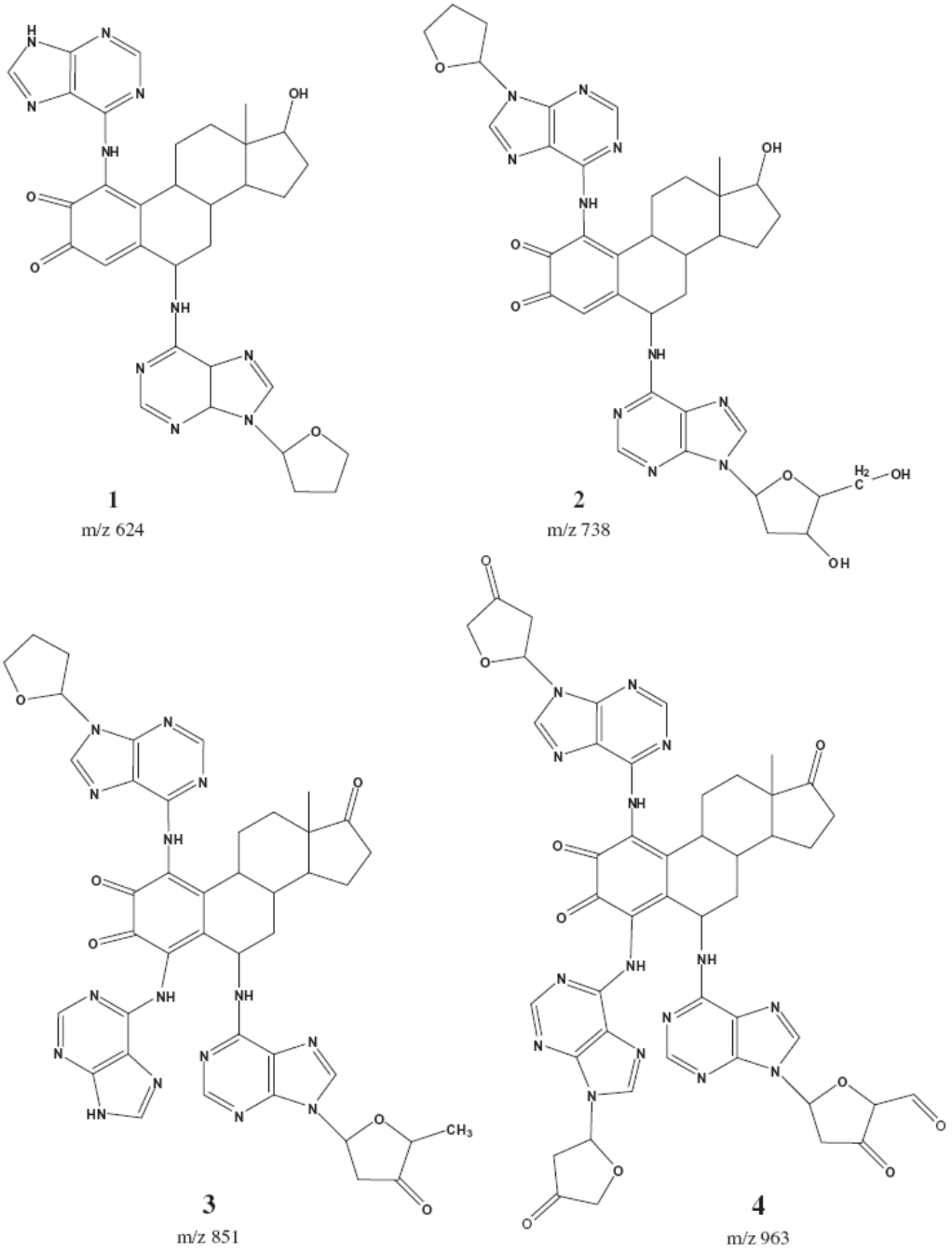


Figure 3



Scheme 1