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# Tumour-like phenotypes in urothelial cells after exposure to antigens from eggs of *Schistosoma haematobium*: An oestrogen–DNA adducts mediated pathway?

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ABSTRACT

Chronic infection with the blood fluke, *Schistosoma haematobium,* is associated with squamous cell carcinoma of the bladder. Previously, it has been shown that soluble extracts of mixed sex adult *S. haematobium* worms (SWAP) are tumourigenic, both in vitro and in vivo. In addition, oestrogen-related molecules in SWAP of *S. haematobium* down-regulate oestrogen receptors (ERs) alpha and beta in oestrogen responsive cells. Moreover, schistosome oestrogens occur in sera of persons with schistosomiasis haematobia and repress transcription of ERs in urothelial cells. Given that eggs of *S. haematobium* are the developmental stage directly responsible for urogenital disease during schistosomiasis haematobia, we suspected that soluble antigens from *S. haematobium* eggs exhibit similar or more potent tumorigenic capacity. Here we investigated the tumorigenic potential of soluble egg antigens (Sh-SEA) of *S. haematobium* and the endocrine system in favouring parasitism by schistosomes. The findings confirmed that 6.25 μg/ml of Sh-SEA was enough to stimulate cell proliferation, reduce apoptosis and increase oxidative stress of Sh-SEA-exposed urothelial cells. In addition, genotoxic effects of Sh-SEA on these cells were determined by using alkaline single-cell gel electrophoresis (Comet). Furthermore, Liquid Chromatography Diode Array Detection Electron Spray Ionisation Mass Spectrometry indicated the presence of catechol-oestrogens in *S. haematobium* SEA. A prospective oestrogen–DNA adduct mediated pathway in *S. haematobium* egg induced bladder cancer is also discussed.



## 1. Introduction

Schistosomiasis is a neglected tropical disease caused by blood flukes of the genus *Schistosoma*. The parasite is transmitted to humans from freshwater snails. Schistosomiasis is one of the major neglected tropical diseases and it is considered the most important of the helminth diseases of humanity in terms of morbidity and mortality. More than 200 million people in 76 countries are infected by schistosomes and 600 million others are at risk of infection. One hundred and twenty million people are considered symptomatic and 20 million have severe disease. The disease may be responsible for an half million deaths per year. No vaccines are available and treatment relies on a single drug, praziquantel (King et al., 2005; Gryseels et al., 2006; Hotez et al., 2008).

Infection is frequently asymptomatic and diagnosis might not be made until a long time after exposure. Adult worms dwell in blood vessels and release eggs that become embedded in the bladder wall, where chronic inflammation, granuloma formation and eventually squamous cell carcinoma (SCC) may be induced. Therefore, *Schistosoma haematobium*, with the liver flukes, *Opisthorchis viverrini* and *Clonorchis sinensis*, is classified as a Group 1 carcinogen by the World Health Organization's (WHO's) International Agency for Research on Cancer (Bouvard et al., 2009), although the cellular and/or molecular mechanisms linking fluke infections with cancer formation have yet to be defined (Sripa et al., 2012).

Bladder cancer is one of the more dire complications of chronic schistosomiasis haematobia (Parkin, 2006; Bouvard et al., 2009; Rollinson, 2009; King, 2010; Kjetland et al., 2012).

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Case report studies indicate that patients with schistosomiasis may develop bladder cancer earlier than uninfected people. The severity and frequency of the sequelae of 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 (Hodder et al., 2000; Herrera et al., 2005). A *S. haematobium*-associated bladder cancer incidence of 3–4 cases per 100,000 has been estimated (Shiff et al., 2006). Felix et al. (2008) showed that the occurrence of transitional cell carcinoma (TCC) of the bladder has supplanted SCC in Egypt following a major decline in the prevalence of urinary schistosomiasis. Such a decline in the pattern of this infection suggests the importance of schistosome-associated bladder cancer that, elsewhere, may be more widespread than is presently thought (Shiff et al., 2010). In recent progress on the understanding of the host–parasite relationship of schistosomiasis haematobia, a draft genome sequence for *S. haematobium* was reported (Young et al., 2012) and a mouse model of *S. haematobium* egg induced immuno-pathogenesis and fibrosis typically found in human urogenital schistosomiasis was described (Fu et al., 2012). It was recently reported that this schistosome is amenable to being cultured in vitro and transformed with nucleic acid probes. Additionally, the presence of an intact and active RNA interference pathway in *S. haematobium* was demonstrated (Rinaldi et al., 2011).

Previously our group reported that soluble extracts of adult *S. haematobium* worms induce tumourigenesis (Botelho et al., 2009b,c). Chinese Hamster Ovary cells (CHO) exposed to whole *S. haematobium* antigens (Sh) induced high cellular proliferation and sarcomas after skin inoculation into nude mice (Botelho et al., 2009c). In addition, Sh-treated CHO cells showed an increased S phase, decreased apoptosis, down-regulation of the tumour suppressor, p27, and upregulation of the anti-apoptotic protein, Bcl-2 (Botelho et al., 2009b). Recent findings also indicate that *S. haematobium* induces the malignisation of the urothelium in CD1 mice (Botelho et al., 2011). However, the cellular and molecular mechanisms implicated have not been fully described. Also, potential parasite carcinogenic components have been investigated by our group (Botelho et al., 2009a, 2010). Four estrogenic molecules have been described in the parasite and in the sera from infected patients. Our results are consistent with the existence of an estrogenic molecule that antagonises the activity of estradiol. We found evidence for this molecule as we identified and characterised, by Liquid Chromatography–Mass Spectrometry (LC–ESI-MS), new estrogenic molecules previously unknown, which were present in the extract of *S. haematobium* worms and sera from schistosome-infected patients.

Here we observed similar molecules in soluble egg antigens (Sh-SEAs) from eggs of *S. haematobium*. These oestrogen-like hormones are known as catechol-oestrogens (Cavalieri and Rogan, 2011). Metabolites of catechol-oestrogens lead to the formation of oestrogen–DNA adducts and genotoxity. Thereafter, loss of DNA adducts can lead to mutations that initiate cancer. Based on the findings presented here, we propose that oestrogen–DNA adducts pathways may underlie the association between *S. haematobium* infection and bladder cancer.

## 2. Material 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 were allowed to acclimate for 1 week 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 six-littermate cages. They were fed standard balanced food and water ad libitum. All of 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 under a 12-h light/12-h dark cycle (from 08:00 h to 20:00 h). 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 samples were 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. Informed consent from patients was obtained. *Schistosoma haematobium* infection was detected by microscopic observation of the eggs in the sediment of centrifuged urine. The eggs were hatched and with the resulting miracidia, snails from a susceptible species, *Bulinus truncatus* strain from Egypt (maintained in our laboratory), were infected. Cercariae were obtained from these snails (Gaubert et al., 1999). Golden hamsters and BALB/c mice were experimentally infected with 100 cercariae; control animals consisted of littermates.

### 2.3. Eggs of S. haematobium and preparation of Sh-SEAs

*Schistosoma haematobium* eggs and adults from an Egyptian strain maintained in the laboratory (Lewis et al., 2008) were obtained from the intestines and livers of infected hamsters, as described (Rinaldi et al., 2011) and stored at −80 °C. Subsequently, eggs were thawed to 4 °C in PBS and lysed by sonication. A soluble extract was obtained by ultracentrifugation of the sonicated eggs. An extract of adult worms was prepared as described (Botelho et al., 2010). The protein concentration of the supernatant was determined using a micro BCA protein assay reagent kit (Viana da Costa et al., 1998), and the supernatant employed as Sh-SEA of *S haematobium*.

### 2.4. Cell lines

HCV29 (normal urothelial) cells were cultured and maintained at 37 °C in a 5% $CO_2$ humidified atmosphere in RPMI medium (Sigma–Aldrich, Saint Louis, MO, USA) with 10% FBS and 1% penicillin/streptomycin (Sigma–Aldrich). Cells were passaged every 5 days. Cells were serum-starved overnight before treatments (Botelho et al., 2012).

### 2.5. Proliferation assay

The CellTiter 96 AQ non-radioactive cell-proliferation assay (Promega, Madison, WI, USA) was used to assess cell viability. The assay employs MTS (3-[4,5,dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4- sulfophenyl]-2H-tetrazolium, inner salt), a tetrazolium compound and the electron coupling reagent, phenazine methosulfate. Viable cells reduce MTS to formazan, which is detected at 490 nm using a spectrophotometer; formazan production is time-dependent and directly proportional to the number of viable cells. HCV29 cells were cultured in 0.1 ml of RPMI media in 96-well flat-bottomed plates. Cultures were seeded at $1 \times 10^4$ cells/well and allowed to attach overnight. After the indicated time of incubation with the appropriate medium, 20 µl of assay reagent were 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 (Botelho et al., 2009b).

### 2.6. Apoptosis

A terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) assay was performed using the in situ cell death detection kit (Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Nuclei were counterstained with DAPI (Roche Diagnostics). The percentage of TUNEL-stained nuclei was evaluated in relation to every DAPI-stained nucleus 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 (Botelho et al., 2009b).

### 2.7. Oxidative stress assay

Oxidative stress was analysed by evaluation of total (GSHt), reduced (GSH) and oxidised (GSSG) glutathione levels. The intracellular levels of GSH and GSSG in Sh-SEA-treated HCV29 cells were evaluated by the DTNB-GSSG reductase recycling assay, as previously described (Costa et al., 2007). After exposure to Sh-SEA, cells were lysed and proteins were precipitated with 5% $HClO_4$. Following centrifugation (16,000*g*, 10 min, 48 °C), the supernatant obtained was used for the determination of GSHt, GSH and GSSG by spectrophotometry at 412 nm.

### 2.8. Comet assay

After treatment, cells were washed twice with chilled PBS ($Mg^{2+}$ and $Ca^{2+}$-free), centrifuged at 78*g* for 5 min and resuspended in PBS. Cell viability was >85% for the tested dose in this study as assessed by using Trypan blue dye-exclusion. The alkaline version of the Comet assay was performed as described by Singh et al. (1988) with minor modifications. Briefly, cells collected by centrifugation (78*g*, 3 min) and suspended in 60 μL of 0.6% low-melting-point agarose (LMA) in PBS (pH 7.4) were dropped onto a frosted slide precoated with a thin layer of 1% normal melting point agarose. Slides were placed on ice for 4 min to solidify the agarose. Coverslips were removed and slides were immersed in lysis buffer (2.5 M NaCl, 100 mM $Na_2EDTA$, 10 mM TrisBase, 0.25 M NaOH, pH 10) for 1 h at 4 °C in the dark. After lysis, slides were placed on a horizontal electrophoresis tank in an ice bath. The tank was filled with 1 mM $Na_2EDTA$, 300 mM NaOH, pH 13 (electrophoresis buffer) to cover the slides. The slides were incubated for 20 min in the dark to facilitate DNA unwinding and alkali-labile site expression.

Electrophoresis was carried out for 20 min at 30 V, 300 mA (1.2 V/cm). The slides were then washed for 10 min with 1 ml of 0.4 M TrisBase, pH 7.5 (neutralising solution). Subsequently, gels were stained with 100 μl of ethidium bromide (20 μg/ml) and covered with coverslips for 20 min. After staining, slides were washed twice with ice-cold, twice distilled water for 20 min.

Slides were coded and examined by a 'blind' scorer using a magnification of 400×. One hundred randomly selected cells (50 per replicate) were examined for each dose. Image capture and analysis were performed with Comet Assay IV software (Perceptive Instruments, Bury St Edmunds, UK); percentage of tail DNA (%T) was the DNA damage parameter evaluated (Kumaravel et al., 2009). The percentage of DNA in the tail is the fraction of DNA in the tail divided by the amount of DNA in the cell multiplied by 100.

### 2.9. Liquid Chromatography Diode Array Detection Electron Spray Ionisation Mass Spectrometry (LC/UV-DAD/ESI-$MS^n$) analyses

The LC/DAD/ESI-$MS^n$ analysis was performed on a Finnigan Surveyor Plus HPLC instrument equipped with a diode-array detector and a mass detector. The HPLC system consisted of a quaternary pump, an autosampler, a degasser, a photodiode-array detector, an automatic thermostatic column compartment and a computer with Xcalibur® software. The mass detector was a Finnigan Surveyor LCQ XP MAX quadrupole ion trap mass spectrometer equipped with an electrospray ionisation (ESI) interface. Control and data acquisition were carried out with the Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA). Nitrogen > 99% purity was used with gas pressure of 520 kPa (75 psi). The instrument was operated in negative-ion mode with ESI needle voltage, 5.00 kV; ESI capillary temperature, 325 °C. The full scan covered the mass range from $m/z$ 50 to 2,000. $MS^n$ data were simultaneously acquired for the selected precursor ion. CID-MS/MS and $MS^n$ analyses were performed using helium as the collision gas with a collision energy of 25–35 eV.

The HPLC used a LiChroCART® C18 column (125 mm × 4 mm; 5 μm particle diameter, end-capped) with the temperature maintained at 25 °C; the mobile phase was composed of (A) 1% (v/v) acetic acid in water and (B) acetonitrile, which had been degassed and filtered. The gradient used 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 A to 100% B. The flow rate was 0.3 ml $min^{-1}$ and split out 200 μL $min^{-1}$ to MS. Spectral data for all peaks were accumulated in the range 200–600 nm. The instrument was calibrated with caffeine (Aldrich, USA), MRFA (tetrapeptide, Thermo Finnigan, USA), and Ultramark 1621 (Lancaster Synthesis, USA) in the mass range of 195–1,821.

### 2.10. Statistical analysis

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

## 3. Results

### 3.1. Schistosoma haematobium Sh-SEA increased the proliferation of urothelial cells in vitro

To begin investigating the effect of Sh-SEA on cell viability and proliferation, HCV29 cells were seeded on 96 well plates, starved overnight, treated with increasing concentrations of Sh-SEA for 24 h, cultivated for 24, 48 and 72 h, and proliferation assessed using a MTS assay (Fig. 1). The growth curve showed that cells incubated in 6.25 μg/ml of Sh-SEA for 24 h proliferated significantly faster and more than control (untreated) cells. We obtained the same results at 48 and 72 h (data not shown). Unexpectedly, higher concentrations of Sh-SEA did not increase cell proliferation and indeed, concentrations of Sh-SEA ⩾ 25 μg/ml caused a reduction in proliferation (Fig. 1).

This outcome suggested that increases in both proliferation and overall survival in HCV29 cells were consequences of exposure to Sh-SEA, at least at the lowest tested concentration of Sh-SEA. Accordingly, this concentration (6.25 μg/ml) was used in the following investigations.

### 3.2. Sh-SEA decreased apoptosis of urothelial cells

To analyse apoptosis, HCV29 cells were seeded on 96 well plates, starved overnight and exposed for 24 h to 6.25 μg/ml of

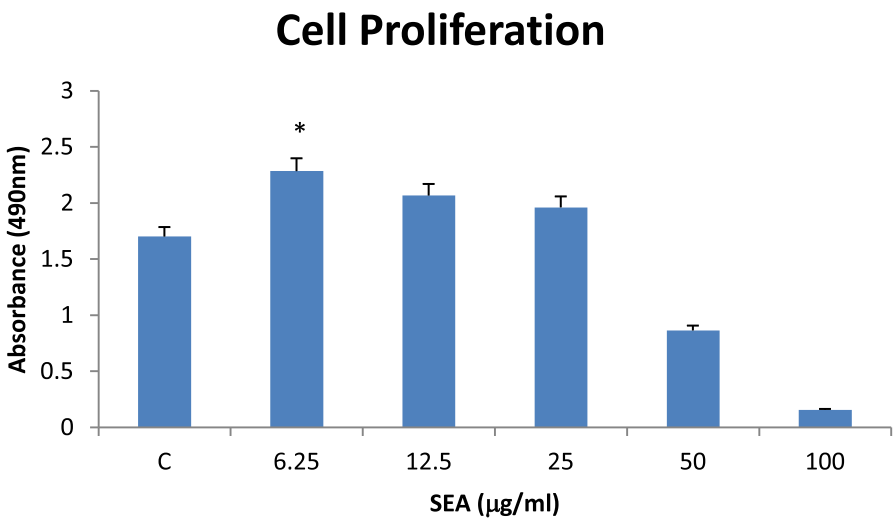


**Fig. 1.** Stimulation of human urothelial cells (HCV29) with soluble egg antigen (SEA) from *Schistosoma haematobium*. Cell proliferation in control (C) or SEA-exposed HCV29 cells at the indicated concentrations for 24 h, harvested 24 h later and analysed by Methosulfate Tetrazolium Salt (MTS) assay. Bars indicate the average of three experiments ± S.D. The asterisk indicates a significant difference ($P \leqslant 0.05$) compared with the control cells.

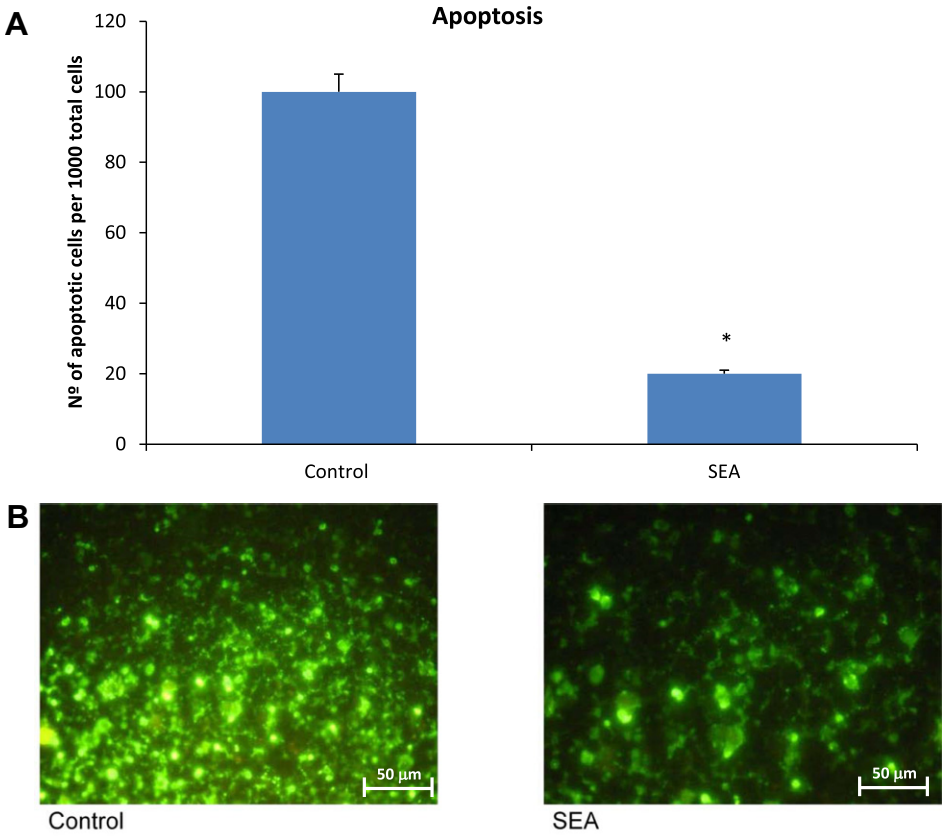


**Fig. 2.** Soluble egg antigen (SEA) from *Schistosoma haematobium* inhibited apoptosis in cultured human urothelial (HCV29) cells. (A) Apoptotic cells observed in control or SEA-exposed HCV29 cells at 6.25 µg/ml for 24 h, cultivated for 24 h and analysed by TUNEL. Bars represent the average of three experiments ± S.D. The asterisk indicates a significant difference ($P \leqslant 0.01$) compared with control cells. (B) Control and SEA-exposed HCV29 cells at 6.25 µg/ml as indicated. Scale bar = 50 µm.

Sh-SEA. Thereafter the cells were cultured for 24 h, harvested and processed for a TUNEL assay (Fig. 2). An increasing number of apoptotic cells per field in controls compared with cells exposed to 6.25 µg/ml of Sh-SEA for 24 h was observed (Fig. 2B). Cell counting resulted in a significant reduction in apoptosis in Sh-SEA-exposed cells compared with controls (Fig. 2A).

### 3.3. Sh-SEA increased oxidative stress of urothelial cells in vitro

Oxidative stress was determined by measuring oxidised glutathione (GSSG). A significant increase in GSSG levels was measured in Sh-SEA-exposed HCV29 cells compared with control cells. Fig. 3 shows the levels of GSSG detected in HCV29 cells after incubation

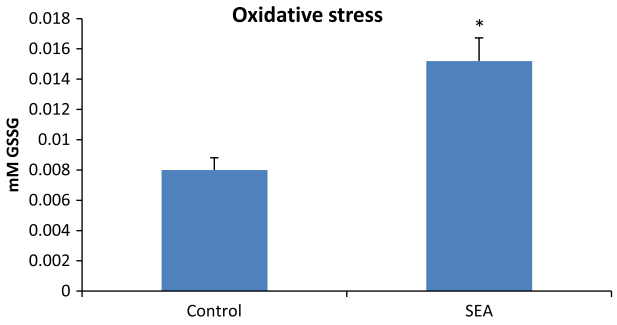


**Fig. 3.** Oxidative stress determined by measuring oxidised glutathione (GSSG). Oxidative stress levels measured in control or *Schistosoma haematobium* soluble egg antigen (SEA)-exposed HCV29 cells at 6.25 μg/ml of SEA. Bars represent the average of three experiments ± S.D. The asterisk indicates a significant difference ($P \leqslant 0.01$) compared with the control cells.

with 6.25 μg/ml of Sh-SEA and in control cells. These results showed that Sh-SEA treatment was able to elicit the alterations in glutathione status.

### 3.4. Induced genotoxicity of urothelial cells in vitro by Sh-SEA

Genotoxicity was detected by a Comet assay as described in Section 2.8. Fig. 4 shows an increase in tail intensity in Sh-SEA-treated cells compared with the control. Less damaged nuclei were observed in the control group of cells compared with cells exposed to 6.25 μg/ml of Sh-SEA (Fig. 4B). A 3.64-fold significant ($P < 0.05$) increase in the percentage of tail DNA was detected in Sh-SEA-exposed cells (47.34 ± 9%) compared with controls (25.19 ± 5%) (Fig. 4A).

### 3.5. Catechol-oestrogens are present in eggs of S. haematobium

HPLC with mass spectrometry was used to identify molecules in samples extracted from Sh-SEA. Fig. 5 depicts UV-chromatograms obtained for biological samples of *S. haematobium* mixed adults (Fig. 5A) and eggs (Fig. 5B). (Given we have previously employed LC–ESI-MS to analyse *S. haematobium* adult extracts (Botelho et al., 2010), extracts of adult worms were included here as a control (Fig. 5A).) In Fig. 6 we can observe the mass spectra ($m/z$) for the principal family of catechol-oestrogens.

## 4. Discussion

Previously we demonstrated that normal cells treated in vitro with *S. haematobium* total antigen display cancer-like phenotypes. Specifically, the cells present rapid uncontrolled division, high resistance to programmed cell death and an atypical capability to migrate (Botelho et al., 2009b) and, when injected into mice with no immune system, lead to the formation of tumours (Botelho 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 inflammation and the development of urothelial dysplasia (Botelho et al., 2011). By contrast, here we characterised the effect of Sh-SEA in human urothelial cells (HCV29) using biological cell approaches typically

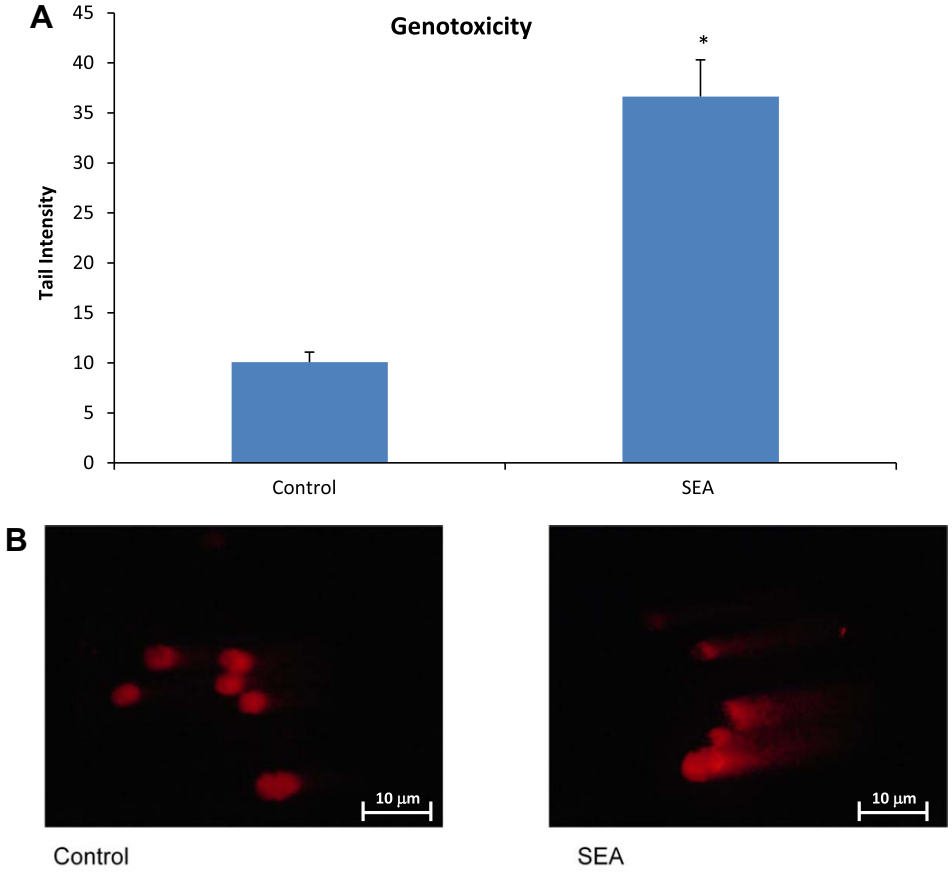


**Fig. 4.** Genotoxicity evaluated by a Comet assay. (A) Tail intensity detected in control and soluble egg antigen (SEA)-exposed HCV29 cells at 6.25 μg/ml of SEA. Bars represent the average of three experiments ± S.D. The asterisks indicates a significant difference ($P \leqslant 0.05$) compared with the control cells. (B) Less damaged nuclei were observed in control cells compared with SEA-exposed cells. Sacle bar = 10 μm.

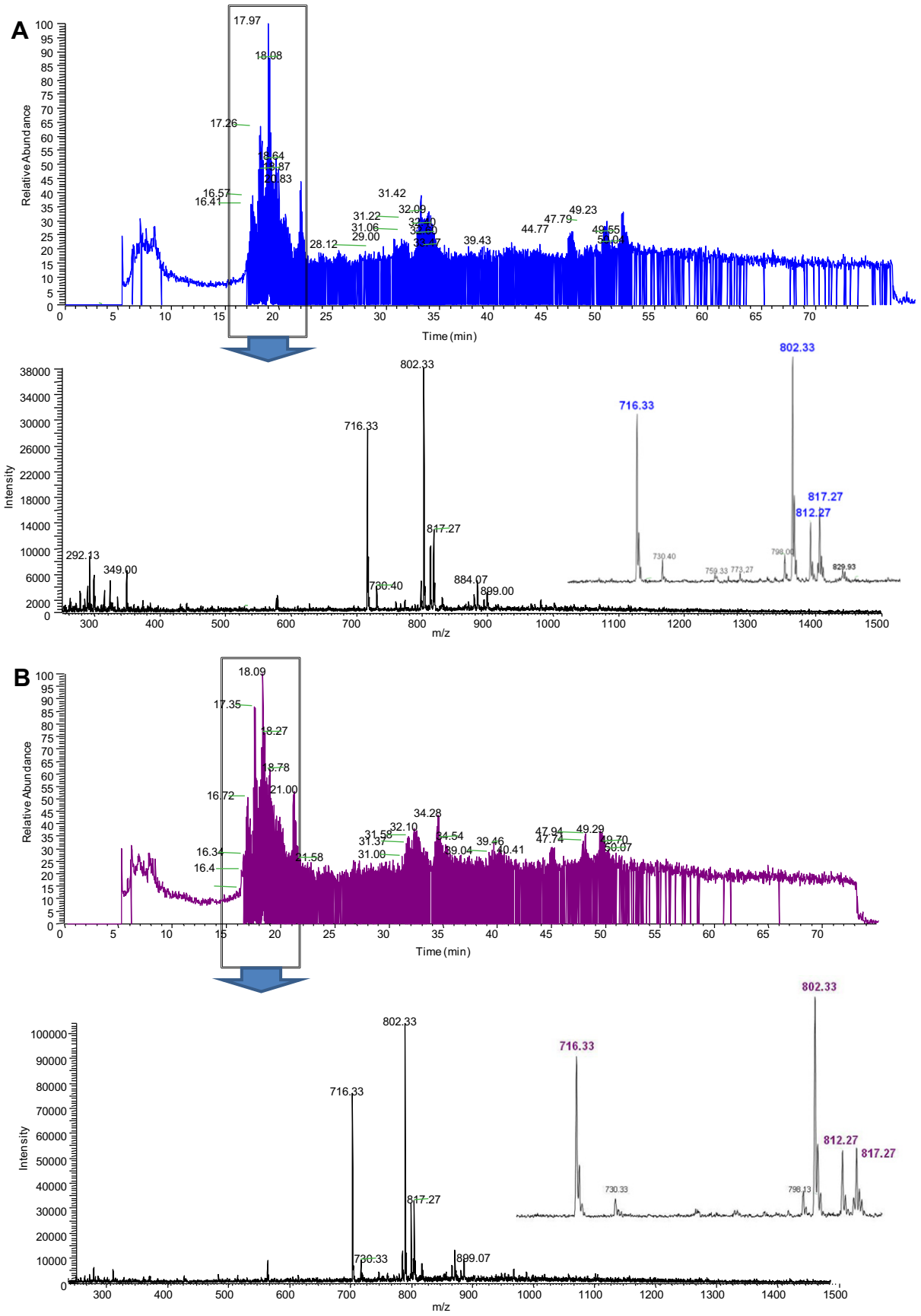


**Fig. 5.** HPLC with MS identifies molecules extracted from *Schistosoma haematobium* adult mixed sex worms (A) and *S. haematobium* eggs (B) as catechol oestrogens.

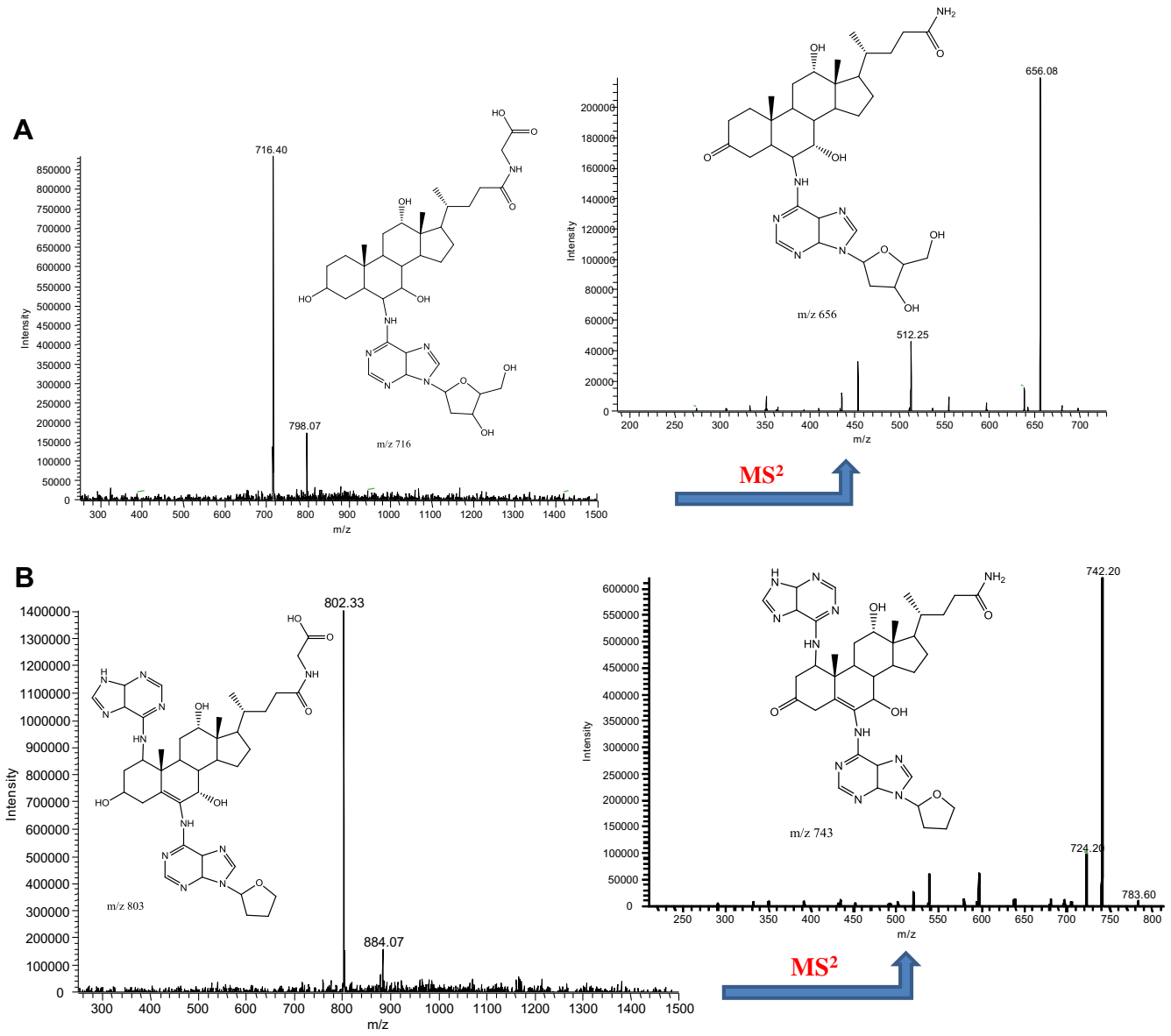


**Fig. 6.** Mass spectra of catechol oestrogens from *Schistosoma* haematobium and molecular structures of components by MS and MS/MS. The molecules are identified by their molecular weight (*m*/*z*). (A) *m*/*z* 716; (B) *m*/*z* 802; (C) *m*/*z* 812 and (D) *m*/*z* 817.

employed in studies of carcinogenesis. Hanahan and Weinberg (2000) presented the following in their findings, "The Hallmarks of Cancer": 'These are biological capabilities acquired during the multistep development of human tumors. The hallmarks constitute an organising principle for rationalising the complexities of neoplastic disease. They include sustaining increasing cell proliferation, apoptosis, inducing oxidative stress and genotoxicity'.

Cell cultures of HCV29 were exposed to Sh-SEA and showed that in a concentration range of 6.25–25 μg/ml, the extract increases proliferation. This observation is in agreement with findings with CHO cells treated with *S. haematobium* (Botelho et al., 2009a,b). It has been shown that prolonged stimulation of excessive proliferation of urinary bladder epithelial cells in rats leads to formation of carcinomas (Otori et al., 1997). However, higher concentrations of Sh-SEA did not stimulate cells to proliferate and indeed concentrations of Sh-SEA $\geqslant$ 25 μg/ml inhibited proliferation. Eggs of *Schistosoma mansoni* secrete a hepatotoxin. Cells infiltrate to surround the newly embolised egg, forming a peri-oval granuloma. In the T cell-deficient mouse, this granulomatous response is lacking, and toxic products released by eggs cause liver damage and death (Abdulla et al., 2011). Thus granulomata protect the host from toxic products of schistosome eggs. Despite the importance of this phenomenon in schistosomiasis mansoni, until recently (Fu et al., 2012), an informative model to analyse the issue in the context of *S. haematobium* SEA was not available. We aim to carry out studies in the future to address this issue.

Apoptotic cell loss in carcinogenesis has been examined by the TUNEL method (Takaba et al., 2000). We also used this method to analyse apoptosis in CHO cells after treatment with *S. haematobium*, where *S. haematobium* dramatically decreased apoptosis in CHO cells (Botelho et al., 2009b). Here, similar phenomena were seen in bladder epithelial cells exposed to SEA of *S. haematobium*.

Oxidative stress has been widely implicated as a mechanism underlying carcinogenesis. Numerous in vitro studies have identified increased reactive oxygen species (ROS) generation as an initiating factor in cancer. The generation of ROS and the resulting oxidative stress may cause a breakdown of membrane lipids, an imbalance of intracellular calcium homeostasis and DNA breakage

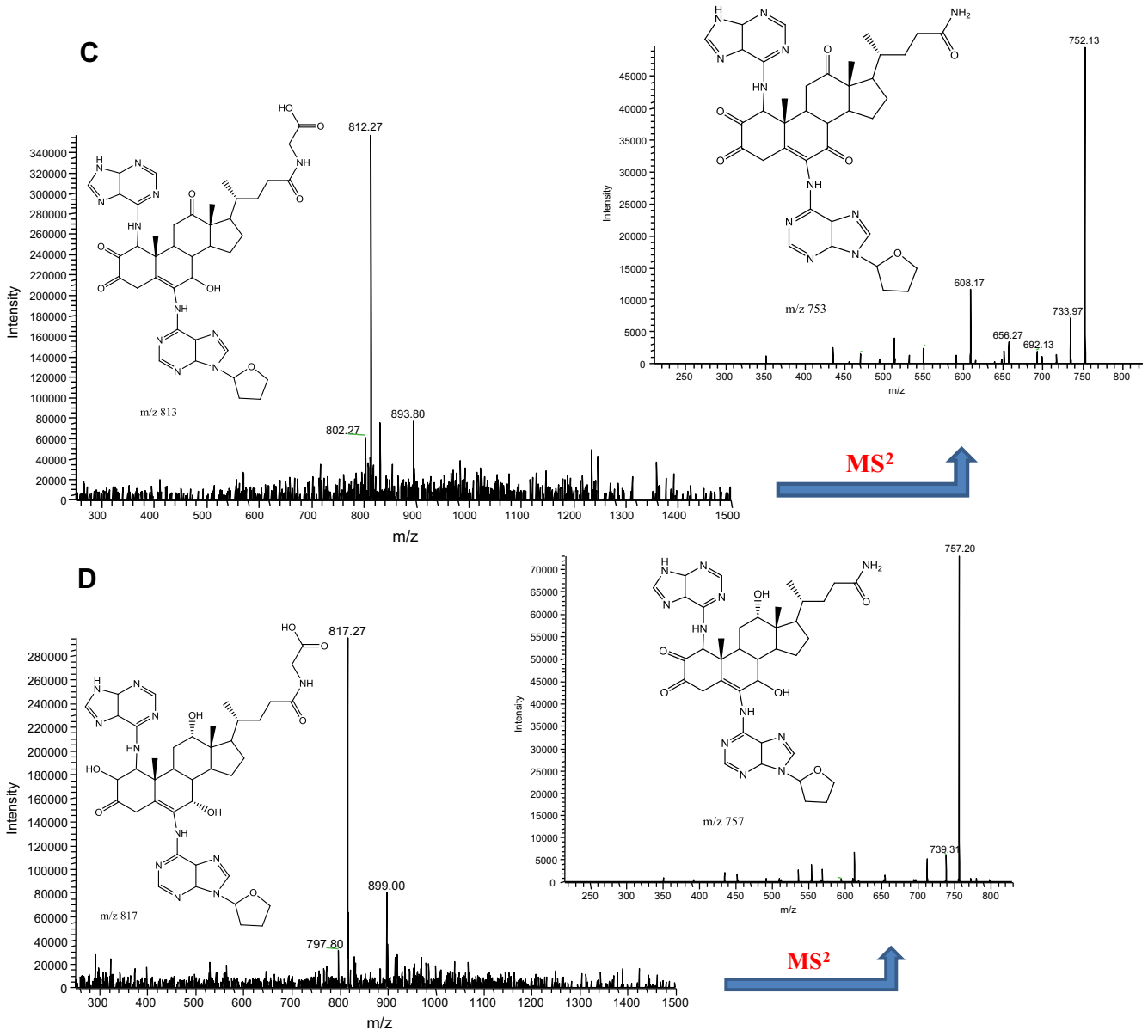


**Fig. 6.** (*continued*)

(Petruska et al., 1991; Clutton, 1997; Shukla et al., 2011). Here we report increased oxidative stress in Sh-SEA-treated cells. Earlier studies showed that *S. 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. 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 (Salim et al., 2008).

The DNA damage response is triggered by the detection of DNA lesions. This response consists of an orderly sequence of signal transduction events that can induce the accumulation of genetic errors which play a critical role in responding to various stresses that cause DNA damage, especially ROS (Matés and Sánche-Jiménez, 2000). We confirmed the genotoxic effects of Sh-SEA on bladder epithelial cells using alkaline single-cell gel electrophoresis (Comet). In the case of *O. viverrini* 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). All of these manifestations facilitate carcinogenesis.

Studying the genotoxic molecular mechanism of Sh-SEA has helped elucidate pathways related to its tumourigenesis. The central hypothesis based on our studies is that genotoxic events and sustained signalling pathway stimulation drive deregulated cell proliferation and anchorage-independent growth; the processes are both required for mutations and progression towards neoplastic lesions, and play a role in Sh-SEA-induced mutagenesis and carcinogenicity. The well-known biological mechanisms, such as the alteration of cell-signalling pathways and induction of DNA damage, play a vital role in neoplasia induction (Huang et al., 2009). The initiation stage of carcinogenesis is mainly characterised by genotoxic processes, which may lead to irreversible changes in the structure of cellular genetic materials. Although DNA repair pathways exist for DNA restoration, however, erroneous repair and extensive DNA damage may cause mutations and ultimately

lead to cell transformation (Huang et al., 2009). Furthermore, since there is a link between DNA damage, mutations and cancer, Sh-SEAs that are potent in causing DNA damage can be regarded as more likely to have an effect on cancer development.

Given the context of the unarguable link between *S. haematobium* infection and bladder cancer, the presence of putative carcinogenic molecules in *S. haematobium* eggs identified here hopefully may have practical consequences for new approaches to control. We have previously identified, by MS in *S. haematobium* extracts and in the serum of infected individuals, four new estrogenic molecules that were formed by reactions of oestrogen-quinones with DNA (Botelho et al., 2010). In the present work we found evidence, as we identified and characterised by MS, similar molecules present in Sh-SEA. The majority of these compounds are catechol-oestrogens. Catechol-oestrogens are formed by hydroxylation on the steroid aromatic ring A. Hydroxylation of both C-2 and C-3 on a steroid ring was apparent and, further, oxidation into an estradiol-2,3-quinone. The genotoxic effects of oestrogen metabolites might be attributed to oxidation of catechol-oestrogens to quinones followed by redox cycling and formation of ROS that in turn react with DNA (Cavalieri et al., 1997; Lu et al., 2007).

To conclude, we anticipate that the findings will contribute to understanding how schistosomiasis haematobia leads to SCC of the bladder. Metabolism of oestrogens and the production of depurinating oestrogen–DNA adducts can be implicated in a pathway underlying *S. haematobium*-promoted host cell DNA damage. The carcinogenic effect of this oestrogen–DNA adduct mediated pathway could explain the link between *S. haematobium* infection and SCC of the bladder. Furthermore, LC/UV-DAD/ESI-$MS^n$ emerges as an important tool to address eventual correlations between oestrogens and *S. haematobium*-associated bladder cancer. We recommend that future studies assess activities of specific catechol-oestrogens identified in schistosome eggs. We plan to follow this route using catechol-oestrogens purified from eggs of *S. haematobium* and/or synthetic versions of these putative carcinogens. In addition, studies utilising RNA interference to silence components of oestrogen catabolism pathways such as schistosome estradiol 17beta dehydrogenase and other catalysts should be informative (Rinaldi et al., 2011; Young et al., 2012)

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