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Response of two vector snail species to infectivity with compatible and incompatible Schistosoma parasites

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ABSTRACT

Schistosomiasis remains one of the most prevalent parasitic infections and has significant economic and public health losses in many developing countries. Understanding the host/parasite interactions is important, since questions arise concerning the susceptibility of snails to infection by respective trematodes and their specificity and suitability as hosts for continued parasite development. Thus the aim of this research is to extend our knowledge about the biological basis of the snail/parasite relationship with the hope of finding novel ways to disrupt the transmission of this disease. In the current research the compatibility/incompatibility of two types of snails,Biomphalaria alexandrina and Bulinus truncatus with their target and non-target miracidia (Schistosoma mansoni and Schistosoma haematobium) was investigated histopathologically and also by identifying some host defense mechanisms against the invading parasite by biochemical analyses through the measurement of lipid peroxides, and the antioxidant enzymes glutathione reductase, superoxide dismutase and catalase in these snails. The results showed that the parasites invading the incompatible snail species were immediately recognized by the host hemocytes and encapsulated at an early stage of snail penetration , while those infecting the compatibile snails were well developed as mother sporocysts. The obtained data also demonstrated that lipid peroxides were increased in snails exposed to the non-compatible parasite while the antioxidant enzyme levels were elevated in snails exposed to the compatible parasite indicating the capability of the respective parasites to overcome the defense mechanisms generated by its host. The infection rate between each type of snail and its compatible parasite was higher than with the non-compatible parasite. Results of these experiments strongly support the hypothesis that endogenous expression and regulation of larval antioxidant enzymes serve a direct role in protection against external oxidative stress, including immune-mediated cytotoxic reactions. This may open new areas for investigating new immuno or chemotherapies or vaccines against the enzymes or products that the parasite releases or needs to survive within its host.

Key words: schistosomiasis, compatibility, oxidative stress, antioxidants.

INTRODUCTION

Species of the human blood fluke *Schistosoma* are estimated to infect approximately 200 million people worldwide, resulting in loss of health, vitality and productivity mainly among the world's poorest inhabitants[1]. Since snail intermediate hosts represent an essential part of the flukes' life cycle, an understanding of the strategies used by the intramolluscan schistosome larvae to survive within this host may provide novel approaches for disrupting larval development and thus transmission to humans[2]. Obliigatory parasitism of the snail, without which no-one can be infected, is the reason why this schistosome-snail interaction is a biomedically relevant target for research. Expanding knowledge of this host-parasite system not only inspires hope of reducing the human costs of this insidious disease [3,4], but holds the promise of broadening and deepening our grasp of the origins and evolutionary histories of the strategies exploited by different hosts and parasites to sustain other symbioses. In Nature, a range of compatibilities exist, and the fates of schistosome larvae that penetrate snails of the host species vary from (i) destruction within hours in non-appropriate hosts to (ii) productive infections that yield human-infective cercariae several weeks later in appropriate hosts. The success or failure of the host's recognition system plays the dominant role in determining the outcomes of schistosome-snail encounters, a state which induces a stress causing formation of free radicals[5]. When trematodes enter a snail, they face oxidative stress generated from products of oxidized plasma hemoglobin , or reactive oxygen or nitrogen species (ROS and RNS, respectively) resulting from hemocyte-mediated immune responses [6]. Trematodes do not "lie down", but, to the contrary, they release a veritable cloud of molecules that have come to be known as their excretory-secretory products (ESP) [7]. Many are released when the miracidia metamorphose to sporocysts, these ESP molecules include antioxidant enzymes which are believed to play a critical role in the maintenance of cellular redox balance, contributing to larval survival in their snail host[8].

Several authors have addressed the fundamental difference between susceptibility or resistance of a certain species of schistosomiasis intermediate host snails as the ability of its respective parasite to activate or inactivate the hemocyte migration towards the miracidia [9,10]. In this respect, studies have confirmed that *Biomphalaria alexandrina* strains , intermediate hosts of intestinal schistosomiasis may be either susceptible or resistant to infection by *Schistosoma mansoni*. Also, *Bulinus truncatus*, intermediate hosts of urinary schistosomiasis may be either susceptible or resistant to infection by *Schistosoma haematobium.*

In the present work, the compatibility/incompatibility of both snail types was studied towards either their target or non-target parasite species in order to confirm the biological basis for snail/parasite associations. Also the rates of infection and attraction of each snail species towards the different parasites was elucidated.

MATERIALS AND METHODS

Preparation of snail tissue homogenates:

Shell of snails from different experimental groups were removed , 0.1g tissue was weighed and homogenized in 1ml phosphate buffer pH= 7.1, centrifuged at 4000 rpm for 15 minutes and the supernatant was collected in epindorff tubes and stored at - 20°C. The supernatants were used for different enzymatic analyses.

Determination of total proteins (TP) in tissue homogenate:
Total proteins were assayed in tissue homogenate using Bradford reagent [11]**.** The color formed is measured calorimetrically at wavelength 595 nm.

Determination of lipid peroxidation products (MDA):
Malondialdehyde (MDA), as an indicator of lipid peroxidation was determined in tissue homogenate according to Ruiz-Larrea *et al*., [12] using saturated thiobarbituric acid (TBA) in 10 % perchloric acid. Lipid peroxidation was expressed as unit absorbance of TBARS at 532 nm. MDA level was calculated using coefficient of MDA 1.56×10^5 / M / cm according to Buege and Aust [13].

Determination of glutathione reductase (GR):
Glutathione reductase was assayed in tissue homogenate according to Erden and Bor [14]. Enzyme activity was calculated by applying the equation of John[15] and expressed as mmoles NADPH reacted/min/mg protein**.**

Determination of catalase (CAT) :
Catalase activity was assayed in tissue homogenate according to Lubinsky and Bewley[16] . The disappearance of hydrogen peroxide was monitored by following the decrease in absorbance at 240 nm in spectrophotometer using molar extinction coefficient for hydrogen peroxide of 0.041 mM/cm. Catalase activity was calculated by applying the equation of John [15]. One unit of the enzyme activity is defined as the amount of enzyme that catalyzes the decomposition of 1 mmol of H_2O_2 / min/mg protein.

Determination of superoxide dismutase (SOD):
Superoxide dismutase was assayed in tissue homogenate by a kinetic assay according to Paoletti, et al., [17]. The initial absorbency was recorded and NADH oxidation was followed by measuring the absorbency at 340 nm after 3 minutes. The activity of the enzyme was calculated according to the equation of John [15]. The activity is expressed as unit / mg protein. One unit is determined as the amount of enzyme that inhibited the oxidation of NADH by 50 %.

Histopathological Investigations:
After removal of shells from each group ,snails were fixed in Boiun solution (200 ml picric acid, 120 ml formaline 37%, 20 ml acetic acid) and then cut (4µm) sections and stained with hematoxylin and eosin .For hematoxylin and eosin (H&E) staining sections were stained with hematoxylin for 3 minutes, washed, and stained with 0.5% eosin for an additional 3 minutes. After an additional washing step with water the slides were dehydrated in 70%, 96%, and 100% ethanol, and in xylene before they were embedded in DPX and evaluated by a blinded pathologist [18]**.**

Snail infection experiment:
Two groups of five Juvenile *Biomphalaria alexandrina* snails (3 ± 1mm in diameter).each were exposed individually to 10 freshly hatched miracidia of *Schistosoma mansoni* or *Schistosoma haematobium* respectively obtained from Schistosome Biological Supply Project (SBSP), (Theodur Bilharz Research Institute, Egypt) in vials containing 3ml dechloratedwater for 3-4 hours and calculate the rate of infection .

Two groups of five Juvenile *Bulinus truncatus* snails (3 ± 1mm in diameter) each were exposed individually to 10 freshly hatched miracidia of *Schistosoma mansoni* or *Schistosoma haematobium* respectively obtained from Schistosome Biological Supply Project (SBSP),

(Theodur Bilharz Research Institute, Egypt) in vials containing 3ml decholoratedwater for 3-4 hours (pH=7 , 25^{o}C) and the rate of infection was calculated per minute.

Statistical analysis:
Data were evaluated with SPSS (Statistical Package for the Social Sciences, version 6.0.1, Chicago, IL) software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference [LSD] test. P values of less than 0.05 were considered to indicate statistical significance. All these results were expressed as mean ±S.D for snails in each group.

RESULTS

Data listed in Table 1 demonstrate that *B.alexandrina snails* infected by *Schistosoma mansoni* show a highly significant in the levels of lipide peroxides, glutathione reductase, catalase and superoxide dismutase with 8.14 ±1, 0.59 ±0.14, 0.08±0.01 and 0.22±0.04 respectively compared to control group.

B.alexandrina snails infected by *Schistosoma haematobium* show a highly significant in levels of lipide peroxides and glutathione reductase with 10.89 ±1.38 and 0.31±0.04 respectively compared to control group and show non significant in levels of catalase with 0.06±0.002 compared to control group and low significant in level of Superoxide dismutase with 0.11±0.02 compared to control group.

On the other hand *Bulinus truncatus* snails infected by *Schistosoma haematobium* show non significant in level of lipide peroxides with 9.4±0.74 compared to control group and show a highly significant in the levels of glutathione reductase,catalase and superoxide dismutase with 0.34±0.05, 0.07±0.008 and 0.21±0.04 respectively compared to control group.

Bulinus truncatus snails infected by *Schistosoma mansoni* show a highly significant in the level of lipide peroxides with 13.03±0.97 and show low significant in level of glutathione reductase with 0.18±0.04 and show non significant in levels of catalase and superoxide dismutase with 0.04±0.003 and 0.1±0.008 respectively compared to control group.

The rates of infection of both snail species with the compatible and incompatible parasites are shown in Figs 1&2. From Fig.1 we could deduce the strong compatability between miracidia of *S.mansoni* with the respective snail *B.alexandrina* and weak attraction with the noncompatible snail *B.truncatus.* Fig.2 reflects strong attraction between the miracidia of *S.haematobium* with their compatible *B.truncatus* snails and weaker attraction with the noncompatible *B.alexandrina* snails. The high infection rate of these snails with the non compatible miracidia may be due to the mobility of this species compared to B.truncatus

Histological and Histopathological Observation:
We observed that miracidia of compatible (C) and incompatible (IC) strains of *S. mansoni* and *S.haematobium* penetrate the snail epithelium in a similar manner (same number of larvae, same speed of entry). Nevertheless, drastic differences were evident between C and IC strains after penetration. Parasites from the IC strain were immediately recognized by haemocytes that were in contact with the surface of the parasites and sporocysts were entirely encapsulated post-infection. At this stage, sporocysts were clearly degraded (Fig. 3c and Fig.4c).Germinal cells and other internal structures showed extensive pathological changes . In contrast, miracidia of the C strain remained unaffected and did not undergo encapsulation, no haemocytes were

observed close to the sporocysts. Normal developing Sp structures were observed (Fig.3b and Fig.4b). It is apparent that fast encapsulation of IC strain miracidia after penetration prevents larvae from moving further into the snail tissues. C strain parasites then pursued normal growth and development.

Table 1:Lipid peroxides, glutathione reductase, catalase and superoxide dismutase in *B.alexandrina* and *B.truncatus* infected with either *S.mansoni* or S.*haematobium*

Group	B.alexandrina	Bio-m	Bio-h	B.truncatus	Bul-h	Bul-m
Lipid peroxides mmole / g.tissue	5.38±1.16	8.14±1[a]	10.89±1.38[a]	8.76±0.74	9.4±0.74[c]	13.03±0.97[a]
Glutathione reductase mmole / g.tissue	0.15±0.02	0.59±0.14[a]	0.31±0.04[a]	0.09±0.02	0.34±0.05[a]	0.18±0.04[b]
Catalase mmole / g.tissue	0.06±0.004	0.08±0.01[a]	0.06±0.002[c]	0.05±0.009	0.07±0.008[a]	0.04±0.003[c]
Superoxide dismutase mmole / g.tissue	0.08±0.01	0.22±0.04[a]	0.11±0.02 [b]	0.084±0.003	0.21±0.04[a]	0.1±0.008[c]

Data are expressed as mean± SD of snails in each g roup Anova at P≤0.05.
[a]P < 0.0001 when compared with normal group.
[b]P< 0.05 when compared with normal group.
[c]P < 0.01 when compared with normal group

Fig. (1): Attraction of *S.mansoni* miracidia towards snails:

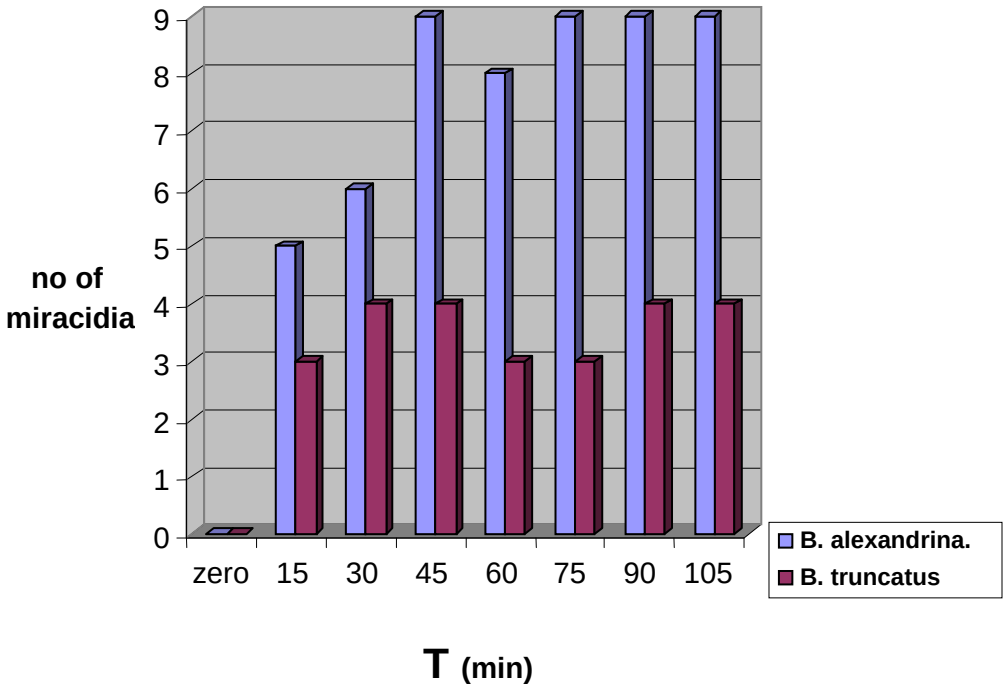
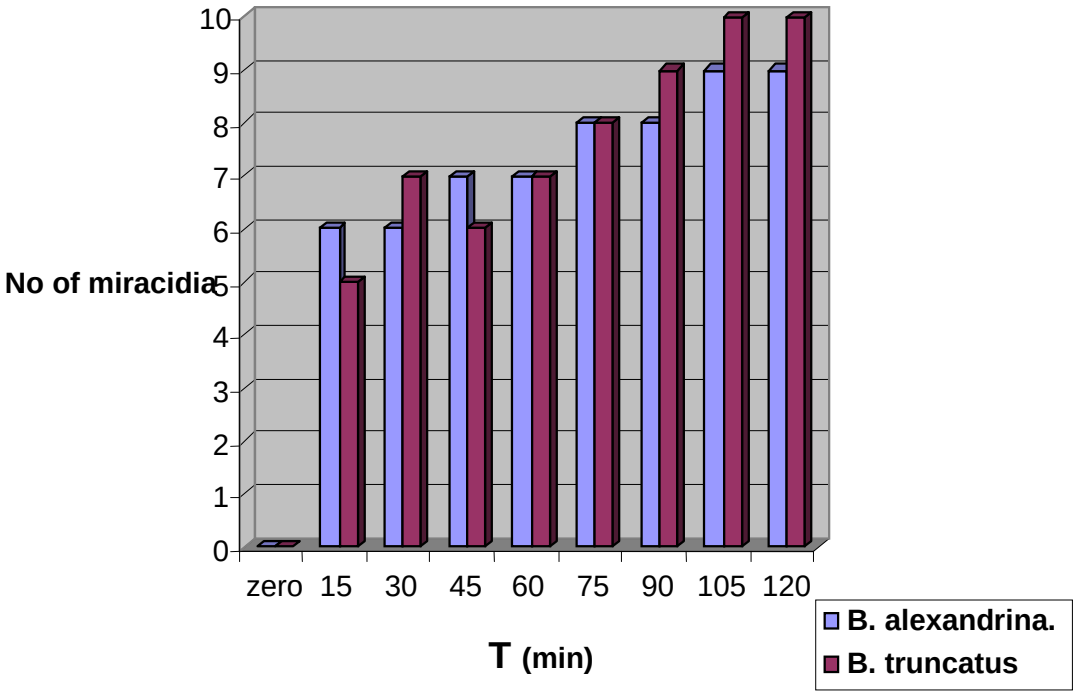


Fig. (2): Attraction of *S.haematobium* miracidia towards snails:



Histological examinations:
-In case of infection with *Schistosoma mansoni*

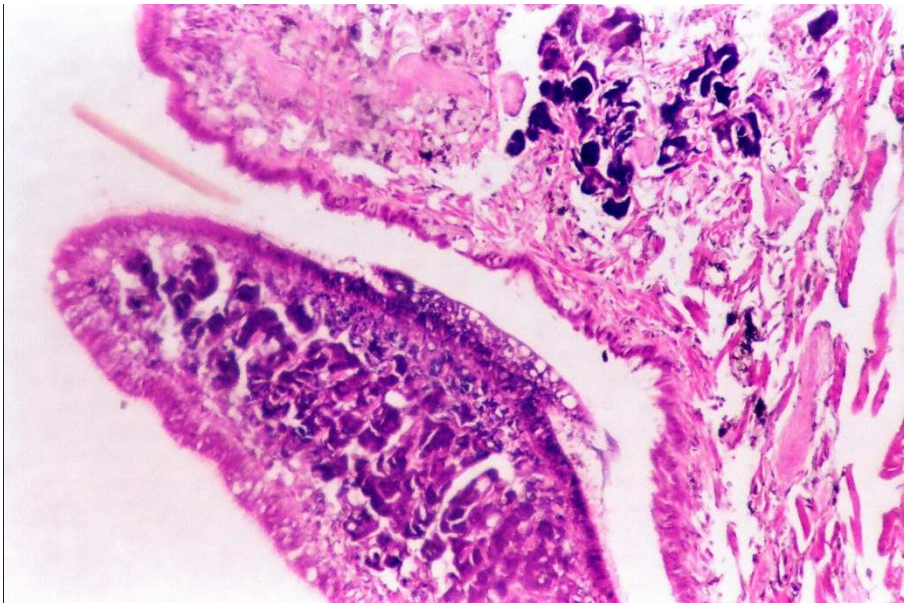


Figure (3a) Histological section of *B.alexandrina* snail showing normal structure of the germ cell.

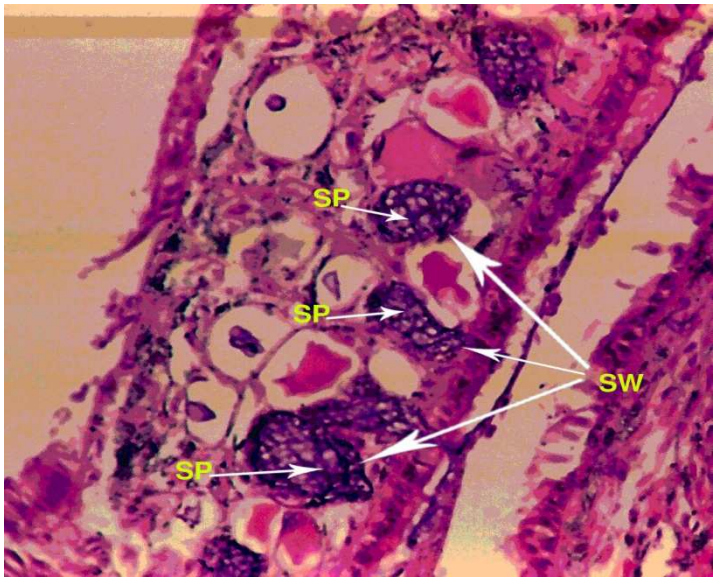


Figure (3b)Histological section of *S. mansoni* Sporocysts (Sp) in a compatible (C strain) host–parasite combination located in head-foot tissues of *B. alexandrina* snails at 3^{ed} days post-infection .Normal developing sporocyst structures, sporocyst wall (sw) is intact; no haemocytes present in the vicinity of the sporocyst. (Haematoxylin &Eosin stain (H,EX200).

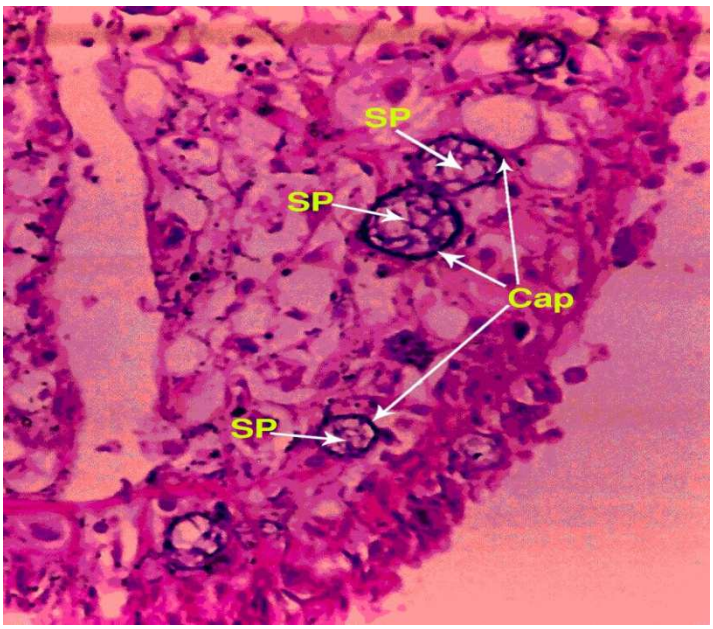


Figure (3c)Histological sections of S. mansoni Sporocysts in an incompatible (IC strain) host–parasite combination located in head-foot tissues of B. truncatus snails at 3^{ed} days post-infection. The sporocyt is encapsulated by hemocytes (cap = capsule), the sporocyst wall (sw) is already clearly degraded and most of sporocyst cells are destroyed. (H,EX200).

-In case of infection with *Schistosoma haematobium*

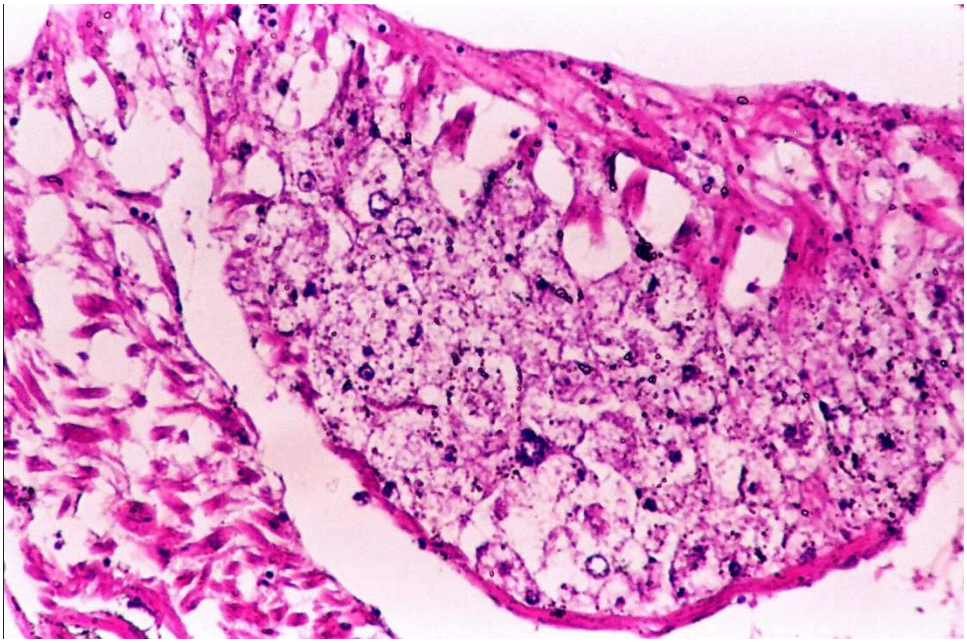


Figure (4a) Histological section of *B.truncatus* snail showing normal structure of the germ cell.

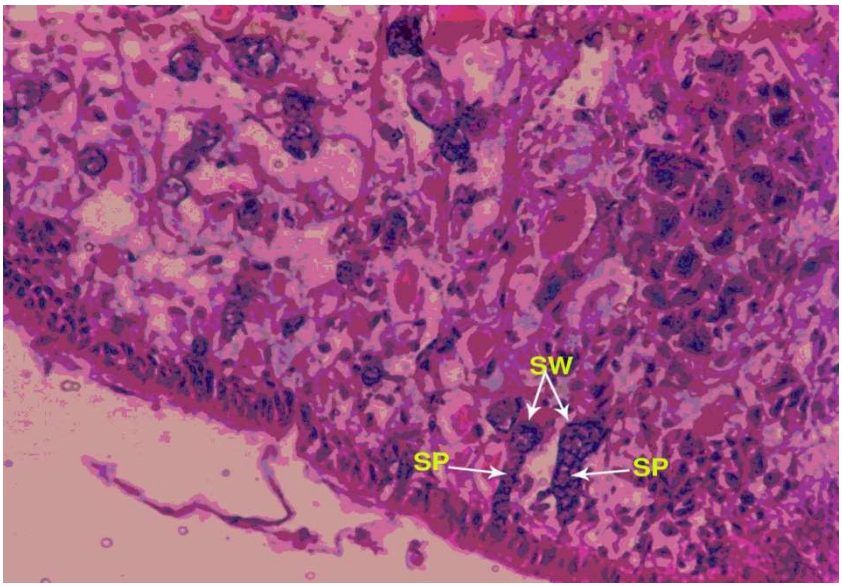


Figure (4b) Histological sections of *S. haematobium Sporocysts (Sp)* in a compatible (C strain) host–parasite combination located in head-foot tissues of *B. truncatus* snails at 3^{ed} days post-infection .Normal developing sporocyst structures, sporocyst wall (sw) is intact; no haemocytes present in the vicinity of the sporocyst. (H,EX200).

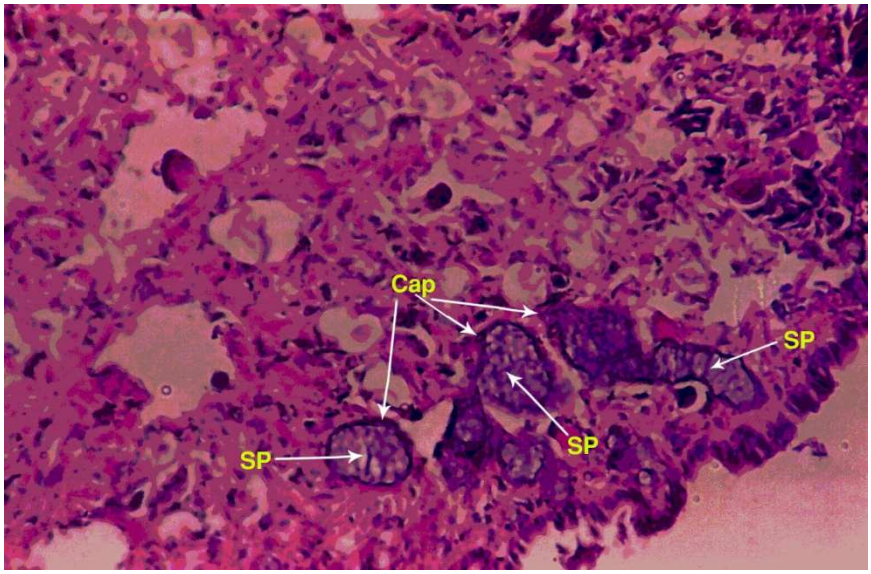


Figure (4c) Histological sections of S.haematobium Sporocysts in an incompatible (IC strain) host–parasite combination located in head-foot tissues of B. alexandrina snails at 3^{ed} days post-infection. The sporocyt is encapsulated by hemocytes (cap = capsule), the sporocyst wall (sw) is already clearly degraded and most of sporocyst cells are destroyed. (H,EX200).

DISCUSSION

The host-parasite relationship is complex and questions remain concerning the susceptibility of snails to infection by the respective trematodes and their suitability as hosts for continued parasite development. The dynamic interaction between molluscs and their trematode parasites leads either to a state of co-existence, in which the trematode thrives and produces subsequent stages of its life-cycle, or to incompatibility, where the trematode is either destroyed and eliminated by the host snail defensive responses or fails to develop because the host is physiologically unsuitable [19,20]. Successful colonization of a compatible snail host by a digenetic trematode miracidium initiates a complex proliferative development program requiring weeks to reach culmination in the form of production of cercariae which, once started, may persist for the remainder of the life span of the infected snail [21].

Hemocytes discover parasites within minutes and can inflict significant damage within a few hours [22]. The capacity to generate ROS, known also by the term ‘leukocyte respiratory burst’ due to its rapid consumption of O_2, is central to the cytotoxic capacities of leukocytes in organisms across the evolutionary spectrum. Once generated, the first ROS of the burst (superoxide, O_2^-) can be metabolized through alternative pathways, some leading to other toxic ROS while others detoxify the products[23].

In the present work ,the antioxidant defense mechanism in snails subjected to either respective or non respective parasites was studied and data showed that lipid peroxide level in *B.alexandrina* snails infected by *S. haematobium* and in *B. truncatus* snails infected by *S. mansoni* was significantly elevated . On the other hand, glutathione reductase (GR), catalase and superoxide dismutase (SOD) activities showed that *B.alexandrina* snails infected by *S. mansoni* and *B. truncatus* snails infected by *S. haematobium* demonstrate a high significant elevation while snails infected by the non compatible parasite showed a non significant difference. It was previously reported that oxidative stress results in formation of highly reactive hydroxyl radical which

stimulates lipid peroxidation [24]. In a corresponding study, Parthasarathy and Joseph [25] stated that oxidative stress induced changes in free radical production and elevated lipid peroxidation in the freshwater Tilapia (*Oreochromis mossambicus*).

As previously reported, the elimination of schistosome parasites by the internal defense of incompatible snails occurs at the first intra-molluscan parasitic stage, namely the mother sporocysts . Also, this period was chosen because during this period it is determined whether the outcome of infection is coexistence with or elimination of the parasite [26]. Farrag [27] studied the levels of different antioxidant enzymes in *B. alexandrina* snail tissue before and after exposure to *S. mansoni* and reported the active role of snail tissue in participation of the antioxidant defense of these snails.

The internal defense system of snails consist of both cellular and humoral components. Circulating hemocytes are the principle line of cellular defense. They can be bound to and kill trematode larva by phagocytosing the syncytial tegument or releasing cytotoxic compounds or both[28]**.**

The current results demonstrate that catalase activity was higher in *B.alexandrina* snails infected by *S. mansoni* and in *B. truncatus* snails infected by *S. haematobium* while *B.alexandrina* infected by *S. haematobium* or *B. truncatus* infected by *S. mansoni* showed a non significant change. Snail hemocytes produce H_2O_2 as an anti-parasite effector molecule, but evidence also strongly supports the presence of an active antioxidant system in early developing *S. mansoni* sporocysts other than catalase for converting H_2O_2, since the latter gene homologues were not found in recent searches of the *S. mansoni* genomic and EST databases [29,30]. This is consistent with previous findings indicating that these parasites must possess alternative means for neutralizing H_2O_2 and other ROS [31,32]. It is thus acceptable that the higher activity level of catalase in snails infected with the compatible parasite arises from the host itself and the excess H_2O_2 that is produced in the cells would be easily detoxified by this enzyme [33], but in non compatible snails, catalase was not able to act efficiently on the surplus H_2O_2 produced and parasite death resulted [34]**.**

The present results indicat that SOD activity in *B.alexandrina* snails infected by *S. mansoni* demonstrate a high significant elevation while those infected by *S. haematobium* showed a non significant change. Also, *B. truncatus* snails infected by *S. haematobium* demonstrate a highly significant elevation while those infected by *S. mansoni* showed a non significant change.

SOD catalyzes the dismutation of superoxide anion ($O_2^{\bullet}$) into hydrogen peroxide (H_2O_2) and O_2. H_2O_2 is the most toxic oxygen species for *S. mansoni* sporocysts . But, SOD also has a peroxidative activity that uses its own dismutation product H_2O_2 as a substrate to produce the hydroxyl radical ($^{\bullet}OH$) [35]**.** Using its peroxidative function, SOD could inactivate H_2O_2 and produce •OH that is less toxic for the sporocysts [9]. This in turn may explain the higher activity of this enzyme in the snails exposed to the compatible parasite.

In the last years, many aspects of the interaction between the digenetic trematode larvae and the internal defense system of molluscs have been elucidated. Nevertheless, the possible mechanisms responsible for destruction of the majority of larvae in resistant or non compatible snails remain to be totally understood. The results reported up to now suggest that the hemocyte could be the effector element in the destruction mechanism of trematodes, being directly involved in the death of some encapsulated parasites or in the production of soluble factors which could be cytotoxic . The majority of the authors [36-38] agree that the snails defense

generally occurs by means of destruction, total or partial, of the primary sporocyst at the first few hours following the penetration of the miracidium.

Briefly, the interaction of snail hemocyte with Schistosoma larvae (miracidium) could be of concern for evaluation of cellular immunity of this snail against infections and could affect schistosomiasis programme control.

The snail infection rate varies widely according to schistosome species and strains, and is, to some extent, related to the snail host-parasite compatibility.

The rate of infection of *B.alexandrina* snails with their compatible parasites (*S.mansoni*) reached 90% .Infection of the same parasite with the incompatible snail *B.truncatus* recorded 40% .This was previously confirmed by Frandsen[39] who found infection rates of *B. alexandrina* snails with *S. mansoni* from Egypt to be about 50-90% and Yousif et al.,[40] who exposed *B.alexandrina* to *S. mansoni* and recorded 100% infection rates. On the other hand, Reda et al.,[41] found that the infection rate of *B.alexandrina* increased up to 88.2% while *B.truncatus* did not**.**

The rate of infection of *B.truncatus* snails with their respective parasite (*S.haematobium*) reached 100 % .This was confirmed by Frandsen [42] who found the rate of infection of *B.truncatus* with *S.haematobium* to be about 62 %-100 %. Infection of the same parasite with the noncompatible snail *B.alexandrina* recorded 90 % and this high infection may be due to the mobility of this species (*B.alexandrina*) compared to *B.truncatus*.

The histopathological investigation in either *B.alexandrina* or *B.truncatus* snails subjected to respective and non respective parasite showed that parasites from the incompatible strain were immediately recognized by haemocytes that were in contact with the surface of the parasites and sporocysts were entirely encapsulated post-infection. At this stage, sporocysts were clearly degraded. In contrast, miracidia of the compatible strain remained unaffected and did not undergo encapsulation, no haemocytes were observed close to the sporocysts and parasites then pursued normal growth and development. This is strongly in accordance with previous findings that light microscopy indicated that hemocytes migrate towards miracidia, after which very thin cytoplasmic extensions develop around the miracidia and finally the *S. mansoni* larva is completely surrounded. The present observation corresponds to early stages of encapsulation classically observed in infected mollusc tissues [43].

The results showed that miracidia penetrated into both the compatible and non compatible snails equally, but hemocytes of the resistant or incompatible snails encapsulated the parasites more quickly and at a higher rate than those from the susceptible strain [44]**.** Resistance can take several forms, occurring because the parasite is not attracted to the snail, cannot penetrate it, penetrates the snail then degenerates immediately or after some development, or is actively destroyed by the snail defence system after penetration [45].

In in vitro studies, hemocytes from resistant snails exhibited greater phagocytic activity than those from susceptible snails [10] **.** In compatible interactions, the parasite penetrates and develops normally within the snail, giving rise to the next parasite stage, the cercariae. Alternatively, in incompatible interactions, the larval trematode penetrates but is immediately recognized as non-self, encapsulated and destroyed by the mollusk's internal defense system [46]**.**

Recent studies based on proteomics of either snail or parasite protein extracts are beginning to reveal key molecules that may play a role in snail/schistosome compatibility [47]**.** A stress response , manifested by modulation of gene encoding heat shock protein 70 may also underlie the snail / host encounter [48]. The authors also reported that heat shock protein 70 and reverse transcriptase are induced early in compatible susceptible snails but not in non compatible ones.

It could be concluded that our findings show a clear link between the oxidant and antioxidant levels between two species of mollusc snails, with their compatible and non compatible parasites, which presumably results from sympatric co-evolution.

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