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Flow Cytometric Analysis of Cell Cycle and apoptosis-regulating proteins in Tissue of Biomphalaria alexandrina Snails.

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ABSTRACT

The present study was designed to understand the host parasite relationships between *Schistosoma mansoni* and its intermediate host, *Biomphalaria alexandrina* snail, using flow cytometric analysis of the cell cycle: gap one stage (GO/1), synthesis stage (S-phase%), and gap two stage (G2/M); and apoptosis-regulating proteins: p53, and Bcl-2. *Biomphalaria alexandrina* snails were exposed individually to miracidia of *Schistosoma mansoni*; and classified into susceptible group (shed cercariae), resistant group (failed to shed cercariae) and control group. In susceptible snails, cell cycle analysis showed significant (p<0.01) decrease in GO/1 and G2/M phases (%) while S-phase% was significantly (p<0.01) increased as compared to both control and resistant snails. P53 protein expression (%) showed significant (p<0.01) increase while Bcl-2 levels showed significant (p<0.01) decrease as compared to both control and resistant snails. In resistant snails, cell cycle analysis showed significant (p<0.05) decrease in GO/1 phase of cell cycle (%), in addition to significant (p<0.01) decrease in S-phase% as compared to control. p53 analysis showed significant (p< 0.01) increase while Bcl-2 analysis showed significant (p< 0.05) decrease as compared to control. We concluded that in susceptible *Biomphalaria alexandrina* snails, *Schistosoma mansoni* infection can modify the normal progression of the cell cycle and induce an apoptotic process.

Keywords: *Biomphalaria alexandrina* snails; *Schistosoma mansoni;* flow cytometry; cell cycle; p53; Bcl-2.

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INTRODUCTION

Schistosomiasis is one of the world's major diseases, and is a major source of human morbidity [1]. It affects more than 200 million people in about 74 countries mainly in tropical and subtropical regions [2]. Schistosomes are the parasitic flatworms that cause schistosomiasis and two species, *Schistosoma mansoni* and *Schistosoma haematobium,* are responsible for the majority of human infections. *S. mansoni* infection is closely related to the existence of its intermediate, fresh water, snail host of the genus *Biomphalaria* and these snails serve as obligatory hosts for the larval stage, and *Biomphalaria alexandrina* (*B. alexandrina*) has historically been implicated in the transmission of *S. mansoni* in Egypt [3]. Interactions between snails and schistosomes are complex and it is important to identify those factors involved in the intricate balance between the snail internal defense system (IDS) and trematode infectivity mechanisms that determine the success or failure of an infection [4].

The cell cycle is a set of events responsible for cell duplication. The inter-phase period of the cell cycle can be divided into three stages: gap one stage (GO/1), synthesis stage (S-phase), and gap two stage (G2/M) [5]. Apoptosis plays an important role in host pathogen interactions and immune defense [6]. Activation of apoptosis process can enhance the immune defense of mollusks and prevent inflammatory reactions in infected or damaged tissues [7]. The cell cycle and apoptosis may be linked as apoptosis can occur at any phase of the cell cycle [8]. In addition, several studies indicate that apoptosis-regulating proteins can have an impact on the cell cycle. P53 plays an important role in cell cycle regulation in addition to encoding an essential protein involved in the growth and regulation of cell proliferation and DNA damage control response by promoting apoptosis [9]. While, Bcl-2 has an inhibitory effect on cell death, thus prolonging cell life by inhibiting apoptosis and its over expression may result in cell proliferation [10].

It is well established that flow cytometry study has been considered as a fast and objective method that allow analysis of almost all cellular functions and parameters. In addition, almost all reagents and fluorescent dyes that have been developed to investigate different cellular activities can be successfully used in invertebrates [11]. From this point of view, our study aimed to evaluate the cell cycle and apoptosis-regulating proteins (p53, and Bcl-2) in tissue of *B. alexandrina* snails during cercarial shedding using flow cytometric analysis.

MATERIALS AND METHODS

Snails and Infection

B. alexandrina snails used in this study were obtained from the laboratory-bred stock of the Medical Malacology Laboratory, Theodor Bilharz Research Institute (TBRI). Laboratory bred *B. alexandrina* snails (3-5 mm in shell diameter) were exposed individually to ten *S. mansoni* miracidia in the presence of 2 ml of de-chlorinated aerated tap water (DTW) under florescent light at temperature (25±1ºC). They remained in contact with miracidia over night and then transferred to their original aquaria, at laboratory temperature (25±1ºC) throughout the infection process. Snails were kept in darkness at 26ºC for about four weeks [12-14]. Thirty days post infection; snails were checked individually for the cercarial shedding [15, 16]. Any snails in which the cercariae were observed were considered infected, and those that did not shed cercariae were considered uninfected.

In this study, snails were divided into three groups (100 snails each), (i) **susceptible group**: snails that shed cercariae; (ii) **resistant group**: snails that failed to shed cercariae; (iii) **control group**: snails not exposed to miracidia. Ten experimental reading were collected from tissue of snails after 40 days (during cercarial shedding) post exposure to *S. mansoni* miracidia, then analyzed and examined by flow cytometry.

Sample Preparation

Fresh soft tissues of the snail were washed with isotone tris EDTA buffer, [3.029 gm of 0.1 M tris hydroxymethylaminomethane (cat. No. T-1378, sigma chemical company), 1.022 gm of 0.07 M sodium chloride (ADWIC) and 0.47 gm of 0.005 M EDTA (cat. No. E-6758, sigma)]; and homogenized in 250 ml distilled water,

pH 7.5. A glass homogenizer was then used to grind the tissue, and the cell suspension was centrifuged at 1800 rpm for 10 min, and the fresh supernatant was decanted.

Flow Cytometric Analysis

Flow cytometric analysis using FACS (flow activated cell sorter) Calibur Flow Cytometer (Becton Dickinson, Sunnyvale, CA, USA) equipped with a compact air-cooled low power 15 mW Argon ion laser beam (488 nm). The average number of evaluated nuclei per specimen was 20.000 and the number of nuclei scanned was 120/s. DNA histogram derived from flow cytometry was obtained with a computer program for Dean and Jett mathematical analysis [17].

Cell cycle analysis: 200 µl cell suspension mixed with 1 ml propidium iodide; samples were filtered (12 x 75 mm, Cat. No. 2058, Falcon Co), then run in the flow cytometer within 1 hour after addition of propidium iodide (PI). The percentage of cells in each phase (G0/G1, S and G2/M) of the DNA cell cycle for each sample was calculated by flow cytometry. The quality of a DNA histogram is estimated from the width of the peak of DNA from the cells in GO/1 of the cycle. The PI is typically detected in a defined range of wave lengths that is designated as the FL2 parameter.

P53 and Bcl-2 analysis as described by Brotherick et al. [18]**:** Cell suspension adjusted to a concentration of $1x10^6$ cell/ml in phosphate buffered saline containing 1% bovine serum albumin (PBS/BSA) buffer. Then 100 µl of cell suspension mixed with 5 µl of antibody p53 or Bcl-2, conjugated with fluorescein isothiocyanate (FITC) and diluted 1:5, and incubated for 30 minutes at room temperature. Cells were washed with 2 ml PBS/BSA and suspended in 2 ml of 0.5% paraformaldehyde in PBS/BSA, and the levels of p53 or Bcl-2 were measured by flow cytometry. Fluorescence generated from the FITC is typically detected in a wavelength band designated as FL1 parameter.

After light scattering and fluorescence is converted to electrical signals by the optical and electronic system, the information is converted into digital data that the computer can interpret.

Statistical Analysis

Results are presented as mean ± standard errors (SE). Significant differences between means were evaluated using one-way analysis of variance (ANOVA) followed by Duncan multiple range test. Person Correlation was used to correlate the means. A probability at level of ≤ 0.05 was considered significant [19]. Results carried out using Instate software computer program, VERSION 5.03 (Graph pad, USA) and IBMPC compatible computer.

RESULTS

Cell Cycle Analysis

Flow Cytometric analysis for cell cycle revealed three distinct cell populations in tissue of *B. alexandrina* snails, which were indicative of cells in the G0/G1, S and G2/M phases of the cell cycle (Table1 and Figures 1).

Table 1 Snail tissues DNA cell cycle flow cytometry analysis in control, Susceptible and Resistant groups of B. alexandrina snails.

Parameters	Snail Groups		
	Control	Susceptible	Resistant
G0/G1	89.09±0.76	65.71±1.18**	85.06±0.16 *
S-phase	11.01±0.16	29.69±2.01**	1.37±0.16
G2/M	5.31±0.25	1.47±0.16**	5.75±0.19

Data are expressed as mean ± SE. Significant difference between groups were shown as $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

M1: G0/1 phase %; M2: S phase %, M3: G2/M phase %.

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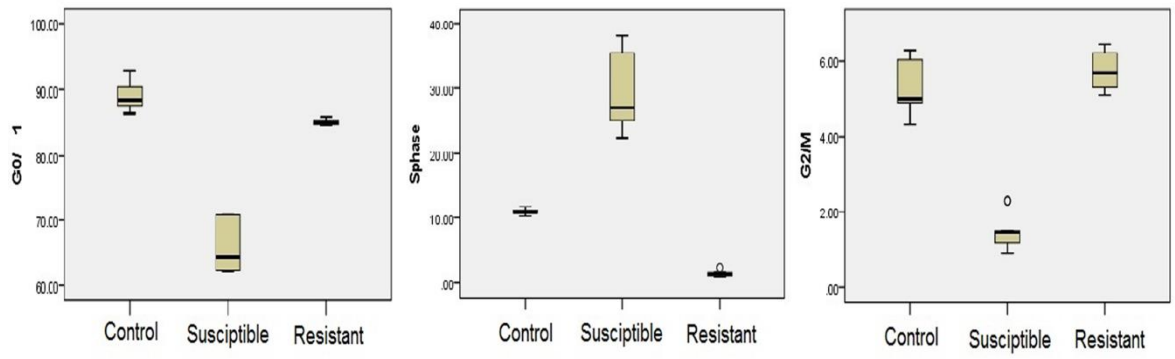

Fig. 1 Box plots of the G0/1, S-phase, and G2/M according to the Control, Susceptible and Resistant. The box represents the inter quartile range; the top and the bottom of the box are respectively the 25th and 75th percentile. The line across the box is the median. The circles represent the outliers.

The susceptible snails showed significant (p<0.01) decrease in levels of both GO/1 and G2/M phases of cell cycle (%) associated with a significant (p<0.01) increase in S-phase of cell cycle (%) as compared to both control and resistant snails.

On the other hand, the resistant snails showed a significant (p<0.05) decrease in levels of GO/1 phase of cell cycle (%), in addition to significant (p<0.01) decrease in S-phase% as compared to control.

P53 and Bcl-2 Analysis

Flow cytometric analysis of p53 showed significant (*P*< 0.01) increase in susceptible snails as compared to both control and resistant snails and also significant (*P*< 0.01) increase in resistant snails as compared to control (Table 2; Figure 2).

Table 2: Flow Cytometric analysis of p53 and Bcl-2 protein expression in Control, Susceptible and Resistant groups of B. alexandrina snails

Parameters	Snail Groups		
	Control	Susceptible	Resistant
P53	13.94±0.21	39.78±0.49**	20.0±0.39
BCl2	42.72±0.72	21.37±0.23**	34.76±0.22*

Data are expressed as mean ± SE. Significant difference between groups are shown as $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

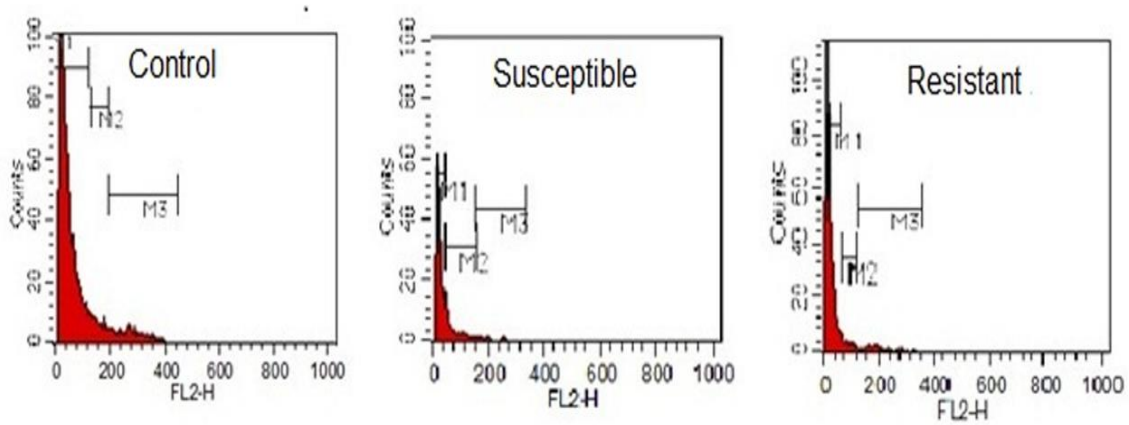

Fig. 2 Flow cytometric analysis, of DNA cell cycle of *B. alexandrina* snail tissue in Control, Susceptible and Resistant groups.

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In contrast, analysis of Bcl-2 showed significant ($p< 0.01$) decrease in susceptible snail tissue as compared to both control and resistant snail tissues and significant ($P< 0.05$) decrease in resistant snail tissue when compared to control (Table 2; Figures 3, 4).

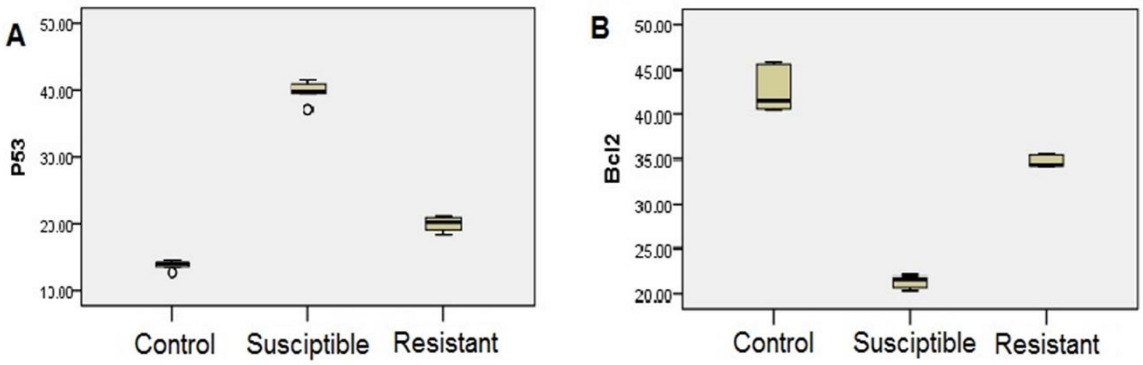

Fig. 3 Box plots of the (A) p53, (B) Bcl2, according to the Control, Susceptible and Resistant. The box represents the inter quartile range; the top and the bottom of the box are respectively the 25th and 75th percentile. The line across the box is the median. The circles represent the outliers.

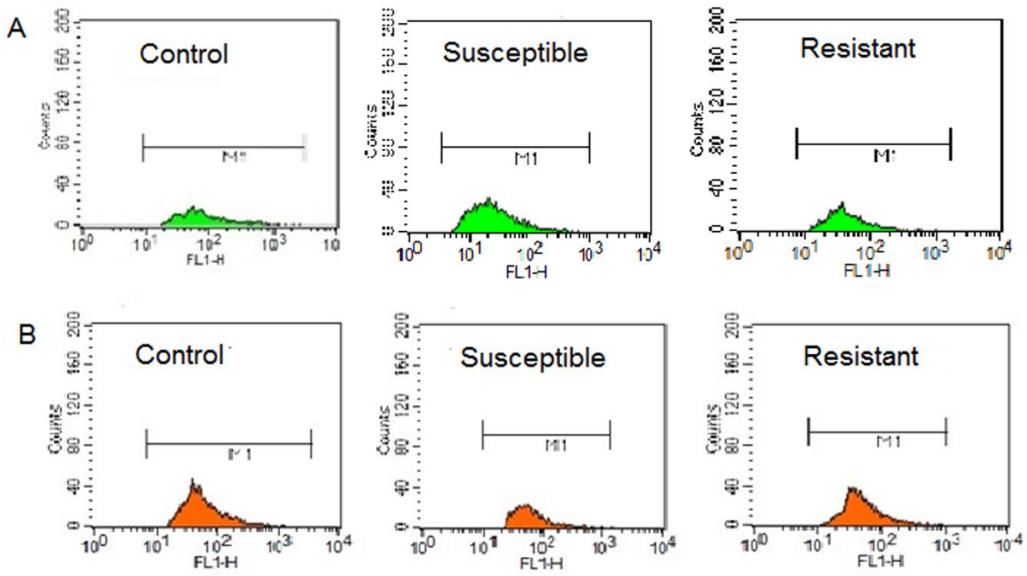

Fig. 4: Flow Cytometric analysis, of (A) p53; (B) Bcl2 of *B. alexandrina* snail tissue in Control, Susceptible and Resistant groups.

Furthermore, a negative correlation ($r= -0.976$, $p<0.001$) was found between p53 and Bcl-2 levels, as shown in Figure 5.

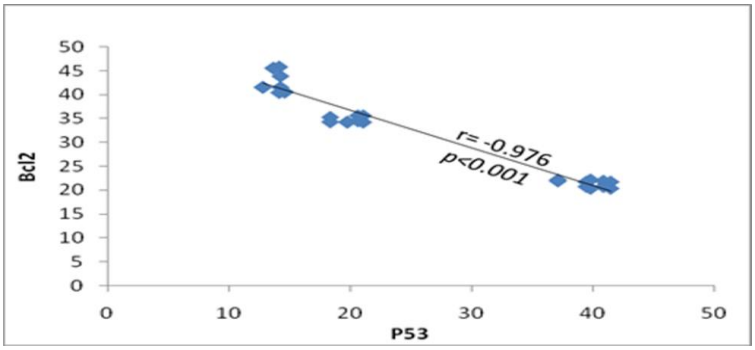

Fig. 5 Correlation between p53 and Bcl-2 levels using flow cytometric analysis (r= -0.976, p<0.001).

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DISCUSSION

The numerous species and strains of *Biomphalaria* vary in their compatibility as a schistosome host such that some display resistance to infection while others are susceptible. Cell cycle is a highly regulated process with numerous checks and balances that ensures a homeostatic balance between cell proliferation and cell death. The timing and order of cell cycle events are monitored during cell cycle checkpoints that occur at the G1/S boundary, in S-phase, and during the G2/M-phases [20]. In the present study, flow cytometric analysis of cell cycle showed a significant decrease in GO/1 and G2/M phases of cell cycle (%) in tissue of susceptible *B. alexandrina* snails as compared to both control and resistant snails. In contrast to the reduction of GO/1 and G2/M, there was a significant increase in the S-phase (%). These remarkable alterations in cell cycle analysis can be attributed to activation of apoptosis due to infection in susceptible snails. These data are consistent with Helal et al., [21] who found an increase of the apoptotic cells in susceptible *B. alexandrina* snails that was associated with a decrease in the difference between condensations and de condensation of DNA. Moreover, the authors indicated the increase in dead cells in susceptible is due to fight between pathogen and snail.

In addition, our data on resistant *B. alexandrina* snails showed significant increase in GO/1 and G2/M phases, as compared to susceptible snails. These data are in agreement with **Helal et al.,** [21] who reported that the percentage of viable cells significantly increased in tissue of resistant *B. alexandrina* snails than susceptible snails and also reported that viable cells were the most common cells that significantly increased than apoptotic, necrotic, and dead cells.

There are many expressed proteins involved in the activation and inhibition of apoptosis including p53 and Bcl-2 [22]. P53 is a well-described mitochondrial apoptotic gene in mollusks [23]. Our data showed that p53 analysis was significantly increased in susceptible snails as compared to both control and resistant snails. These findings are in agreement with previous studies showing that p53 governs the decision between cell survival and apoptosis and its induction can initiate prolonged cell-cycle arrest in G1 and the G2/M checkpoints of the cell cycle [24-26]. Similar results also were obtained by Oren [27] that expressed the major functions of p53 to include cell cycle arrest, DNA repair and apoptosis and demonstrated that, in response to cell damage, p53 is implicated in controlling the G1- to S-phase transition, blocking cell cycle progression at G1 in response to DNA damage.

Regarding resistant *B. alexandrina* snails, our data showed down-regulation of p53 levels as compared to susceptible snails associated with up-regulation of p53 levels as compared to control. Similarly, Helal et al., [21] found increase of the percentage of apoptotic cells in resistant *B. alexandrina* snails than control and attributed this increase to the overcome of the invading pathogenic organism.

It was demonstrated that Bcl-2 proteins are key regulators of mitochondrial apoptosis pathway in response to various intrinsic and extrinsic stimuli [28]. In addition, Bcl-2 plays a role in regulating cell cycle entry. In the present study, flow cytometric analysis of Bcl-2 revealed a significant decrease in susceptible snails as compared to both control and resistant snails. Similarly, Strasser et al. [29] showed that Bcl-2 and Bcl-xL inhibit apoptosis of proliferating cells. The surviving cells undergo cell arrest and accumulate in the G0/G1 phase of the cell cycle. Moreover, our data showed up-regulation of Bcl-2 levels in resistant snails as compared to control. Bcl-2 does not significantly affect growth rates under optimal conditions, but prolongs G1 in suboptimal conditions [30, 31].

Furthermore, our study showed a negative correlation between levels of p53 and Bcl-2. Consistently, Böttger et al. [32] stated that apoptosis can be induced by p53 either via translocation into the nucleus where it upregulates transcription of pro-apoptotic genes or by translocation to the mitochondria where it binds to and inactivates Bcl-2 and other antiapoptotic proteins. Este´vez-Calvar et al., [33] revealed that p53 gene could induce the upregulation of the pro-apoptotic Bax gene that could repress the transcription of the antiapoptotic genes Bcl-2.

In conclusion, our study showed that *S. mansoni* infection can modify the normal progression of the cell cycle and induce an apoptotic process through up-regulation of p53 expression and down-regulation of Bcl2 in susceptible *B. alexandrina* snails. However, resistant snails showed normal cell cycle, down-regulation

of p53 expression and up-regulation of Bcl-2 as compared to susceptible snails. Therefore, flow cytometric analysis can provide a simple, reproducible, and sensitive method for evaluating *B. alexandrina* responses to immunological stimuli. Such knowledge can help in the establishment of novel control strategies and explain new features of the parasite-host relationship through understanding pathways and genes involved in the snail internal defense system.

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