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Analysis of Circulating Heamocytes from *Biomphalaria Alexandrina* Following *Schistosoma Mansoni* Infection Using Flow Cytometry

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ABSTRACT

Background: The internal defense system of snails consists of both cellular and humoral components. Circulating haemocytes are the principle line of cellular defense. The susceptibility of fresh water snails of genus *Biomphalaria* to infection by *Schistosoma mansoni* (*S. mansoni*) is linked to the haemocytes present in the haemolymph. While the humoral component includes lysosomal enzymes, that helps in recognition of pathogens and parasites.

Material and Methods: In the present work, *Biomphalaria alexandrina* (*B. alexandrina*), snails were exposed individually to *S. mansoni* infection; according to their response they were classified into susceptible group (shed cercariae) and resistant group (failed to shed cercariae). Snails not exposed to infection were considered as control group. Flow cytometric analysis was carried out for detecting apoptosis of *B. alexandrina* haemocytes isolated from pooled haemolymph and tissue.

Results: This study is used to discriminate and count the percentage of viable, apoptotic, necrotic, and dead cells in haemolymph of all studied groups. Comparing to control group, we observed that viable cells were the most common cells that significantly decreased in both infected and non-infected groups ($P<0.001$, $P<0.01$ respectively), and showed significant decrease in infected in comparison with non-infected group ($P<0.01$). The haemolymph in infected group showed that dead cells were the most common cells (more than the viable, apoptotic and necrotic cells). At the same time, the apoptotic cells showed decrease in the condensation and de- condensation of DNA. This means failure to restore DNA.

Conclusions: There was a significant variation in the number of the cells between susceptible and resistant snails. In susceptible snails the defense process failed, this was confirmed with increase of the dead cells in the infected haemolymph. While in resistant snails the viable spreading cells described as immunological active, are predominantly found in the haemolymph and tissue.

Key Words: Apoptosis; *Biomphalaria alexandrina*; Flow Cytometry, Immune response.

INTRODUCTION

The internal defense system (IDS) of snails is composed of cellular elements; haemocytes and soluble factors present in haemolymph. They play an important role in the innate immune responses of mollusks and have important factors in fighting snail infections[(1)]. It is responsible for the phagocytosis, cytotoxic reactions, and the synthesis of the humoral factors[(2)]. Humoral factors play a fundamental role in the innate immune responses in mollusks[(2)]. The humoral components of the molluscan immune defense include lysosomal enzymes such as (β- glucuronidase, acid and alkaline phosphatase, lipase, aminopeptidase, lysozyme), lectins including agglutinins, fibrinogen-related proteins (FREPS), and C-type lectins, antimicrobial peptides that aid in recognition of pathogens and parasites by marking them for destruction via opsonizing or directly killing[(3,4)]. Apoptosis of immune cells can play an important role in protection against parasites and pathogens by the innate immune system. Apoptosis is immunologically silent and does not induce inflammation[(5)].

Haemocytes play the prominent role in parasite and pathogen elimination. The haemocytes may be circulating in haemlymph or fixed in tissues. Snail haemocytes are able to the mount a cytotoxic response against parasites[(6)].

The immune response is complemented by the generation of highly reactive oxygen species (ROS). Internal defense system is one of the factors that influence the susceptibility pattern of the snails. This system is

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stimulated by the excretory secretory products (ESPs) of the penetrating miracidia[7]. The ROS were supposed to directly account for the loss of mitochondrial membrane potential (MMP) and for phosphatydylserine exposure at the cell surface membrane, suggesting the execution of an intrinsic apoptotic pathway. Cell surface exposure of phosphatydylserine is an important hallmark of apoptosis. It is a phylogenetically well conserved process, which was demonstrated in a number of vertebrate species and in a few invertebrates[8]. Apoptosis plays a role in host protection against parasites by limiting the spread of the pathogen while preventing inflammatory damage of surrounding tissues [9].

This work was designed to discriminate and count the percentage of haemocytes in an attempt for better understanding of immunological response of *B. alexandrina* to *S. mansoni* infection. The effectiveness of *B. alexandrina defense* system can modulate the properties of infection with *S.mansoni*.

MATERIAL & METHODS

B. alexandrina snails used in this study were obtained from the laboratory-bred stock in Medical Malacology Laboratory, Theodor Bilharz Research Institute (TBRI) Egypt. Laboratory bred *B. alexandrina* snails were exposed individually to ten *S. mansoni* miracidia in the presence of 2 ml of dechlorinated aerated tap water 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[10]. They were kept in the darkness at 26ºC for about four weeks. Fresh lettuce leaves were supplied as food every couple of days and the dead snails were regularly removed. Thirty days post infection; the snails were checked individually for the cercarial shedding[11]. Three groups (300 snails each) were used in this experiment, two groups from these exposed snails were used, the snails that shed cercariae were considered as susceptible to infection (group one) while those failed to shed cercariae were found alive under microscopic examination, these snails were considered resistant to infection (group two) and 300 snails were not exposed to miracidia (group three).Ten experimental reading of haemolymph and tissue of snails were collected after 40 days (during cercarial shedding) post exposure to *S. mansoni* miracidia and then analysed and examined.

Collection of Haemolymph:

Haemolymph samples were collected from all experimental group outlined by **Michelson**[12] via removing a small portion of the shell and inserting a capillary tube into the heart. Haemolymph was pooled from 300 snails collected in ten vial tubes (1.5 ml) each containing 30 snails and kept in ice-bath for analyzed with a cytofluorometer, by using Annexin-V FITC apoptosis detection kit I (BD Pharmagen™).

To measure the de-condensation, 100 ml from the sample was taken in two tubes 12x75 polystyren BD for staining. One treated with EDTA, borate buffer, SDS (sodium dodecideyle sulphate) to measure the de-condensation, then the two tubes were stained with propidium iodide 50 mg/ml buffer (citrate buffer and titron x and propidium) for at least one hour in dark at +4°C until acquisition.

Detection of apoptosis by using flow cytometry

Flow cytometric analysis was carried out for detecting a poptosis of *B. alexandrina* haemocytes isolated from pooled haemolymph and tissue samples from 300 snails in each experimental group using a FACScan flow cytometer (BD-Becton Dickinson, USA). The flow cytometer was set to collect 4000 events; gated regions were set to determine forward scatter (FSC) and side scatter (SSC), based on the size and granularity of cells, and to categorise them into different subpopulations based on their emission of fluorescent signals. Data were analysed by using Cell Quest software (Becton Dickinson).

Apoptosing cells were quantified by a coupled reaction via annexin V-FITC and PI. This method of apoptosis detection was based on the binding properties of annexin V to PS and on the ability of PI to intercalate nuclear DNA.

The percentage of apoptotic haemocyte was assessed from Annexin-V protein binding and propidium iodide staining by flow cytometry[13]. Quantification of the immunolabelling annexin V and PI in

haemocytes, after post exposure to infection, results were expressed as the mean frequency of fluorescent events within the haemocyte population ± SE of the total cell count.

Samples were analyzed by using flow cytometry. This technique takes advantage of a redistribution of plasma membrane phospholipids in the early stages of apoptosis. Phosphatidylserine, which normally residues on the inner membrane leaflet, has been shown to be expressed on the outer membrane as an early feature of apoptosis regardless of initiating stimulus. Annexin V conjugated to FITC will bind specificially to phosphatidylserine and thus can be used to quantify the number of cells expressing phosphatidylserine and undergoing apoptosis. Propidium iodide was used to stain cellular DNA; the cellular membrane must not be intact. These cells are thus determined to be necrotic.

For each haemocyte sample, four distinct sub-populations of haemocytes were found by a method involving the uptake of a combination of dyes (annexin V-FITC and PI). The absence of detectable dye uptake indicated that cells in region LL represented the viable sub-set. The relatively small sub-population with high staining of annexin V in region LR suggested the presence of early apoptotic cells. Cells in region UR were considered to be in a late phase of the necrotic process as they exhibited a high scatter signal of annexin V but still incorporated the DNA dye. When PI was incorporated into the cells, it indicated greater plasma membrane permeability, a loss of membrane integrity. The cells in region UL showing high uptake of DNA dye were considered to be dead.

The data is plotted in two dimensions with frequency on the vertical axis and the magnitude of the variable on the horizontal one. Chromatin condensation and chromatin de-condensation mean were analyzed using FACS caliber flow cytometer. Units of magnitude on the horizontal axis are historically referred to as channels. The histogram represents the relative number of cells that fall into each of the channels representing different signals intensities.

Statistical Methods

All datasets were statistically explored with univariate or multivariate parametric analyses of variance (one- way ANOVA). Before the analyses of results, we checked for normality and homocedasticity of the error terms of the model. A Box-Cox transformation was applied to any data for which observed deviations from group means lacked normality (Fig.1). When heterogeneity among means was detected in multi sample experiments, we used a multiple comparison approach (Tukey test) procedure to compare group means with a control. Interdependance of variables describing relevant cell states or cell populations was assessed by means of the product moment correlation coefficient (r) Spearman coefficient. All statistical analyses were computed by using SPSS/Pc[(14)]. Program version 18.0 to determine the significant of data.

Results

Comparing to control, Immunostaining of haemolymph using Annexin V and propidium iodide showed that: the percentage of viable cells significantly decreased in infected and non-infected groups (P<0.001, P<0.01 respectively). The decreased in infected haemolymph group were more than that in non-infected group (P<0.01). Also, in control and non-infected groups, the percentage of apoptotic, necrotic, and dead were less than viable cells.

Concerning the infected group, the most prominent haemolymph cells were dead cells (65.9%±1.3). This was associated with marked nuclear staining with propidium iodide (Table1, Fig.2, and Fig.4).

Tissue staining revealed the same results with the exception of the infected group, the most prominent cells were necrotic cells (65.9%±0.89).This was also associated with marked nuclear staining PI with annexin V-FITC (Table 2, Fig.3, Fig.5).

Both infected and non–infected groups showed a significant decrease (P<0.01) in difference between chromatin condensation and chromatin de-condensation means when compared to control group. Similar trend was observed in differences between chromatin condensation and chromatin de-condensation mean (P<0.01) when compared infected group to non-infected group (Fig.6, 7, 8).

Correlation analysis revealed a highly significant positive correlation between haemolymph and tissue in control group

(r=0.743, $P< 0.01$); and non-infected group (r= 0.875, $P< 0.01$) (Fig.9)

Table 1: Immunostaining of Annexin V and propidium iodide in haemolymph in all studied groups.

Groups	Type of cells			
	Viable Cells (Mean±SE)	Apoptotic Cells (annexine) (Mean±SE)	Necrotic Cells Dual cells (Mean±SE)	Dead Cells P.I (Mean±SE)
Unexposed (control)	89.65±0.96	4.9±0.77	4.2±0.52	1.3 ±0.16
Exposed (non-infected)	74.50±1.60 [a]	7.1±0.3 [*]	8.3±1.0 [a]	10.1±1.0 [a]
Exposed (infected)	3.50±0.28 [ab]	6.1±0.58 [a]	24.5±1. 2 [a,b]	65.9±1.3 [a,b]

Data expressed are% of mean ± standard errors (SE).
[a]*P*<0.001,[b]*P*<0.01 relative to groups (control and non-infected) respectively,*P<0.05 relative to control group.

Table 2: Immunostaining of Annexin V and propidium iodide in snail tissue in all studied groups.

Groups	Type of cells			
	Viable Cells (Mean±SE)	Apoptotic Cells (annexine) (Mean±SE)	Necrotic Cells Dual cells (Mean±SE)	Dead Cells P.I (Mean±SE)
Unexposed (control)	84.18±1.00	1.2±0.16	5.29±0.79	9.01 ±1.23
Exposed (non-infected)	71.16±0.72 [a]	7.9±0.58 [a]	9.8±1.25 [a]	11.4±0.53 [a]
Exposed (infected)	4.51±0.22 [ab]	6.2±0.59 [a]	65.9±0.89 [a,b]	23.9±0.65 [a,b]

Data expressed are % of mean ± standard errors (SE)
[a]*P*<0.001 [b]*P*<0.01 relative to groups (control and non-infected) respectively.

Table 3: Condensation and de-condensation mean in all studied groups.

Group	Condensation (Mean ± SE)	De-condensation (Mean ± SE)	Difference (Mean ± SE)
Unexposed (control)	106.4 ± 0.70	143.8 ± 0.82	37.4 ± 1.2
Exposed (non-infected)	136.9 ± 0.64	150.3 ± 0.73	13.4 ± 0.476 [a]
Exposed (infected)	142.6 ± 0.47	144.9 ± 0.48	2.3 ± 0.3 [a]

[a]*P*<0.01 Relative to groups (control and non-infected).

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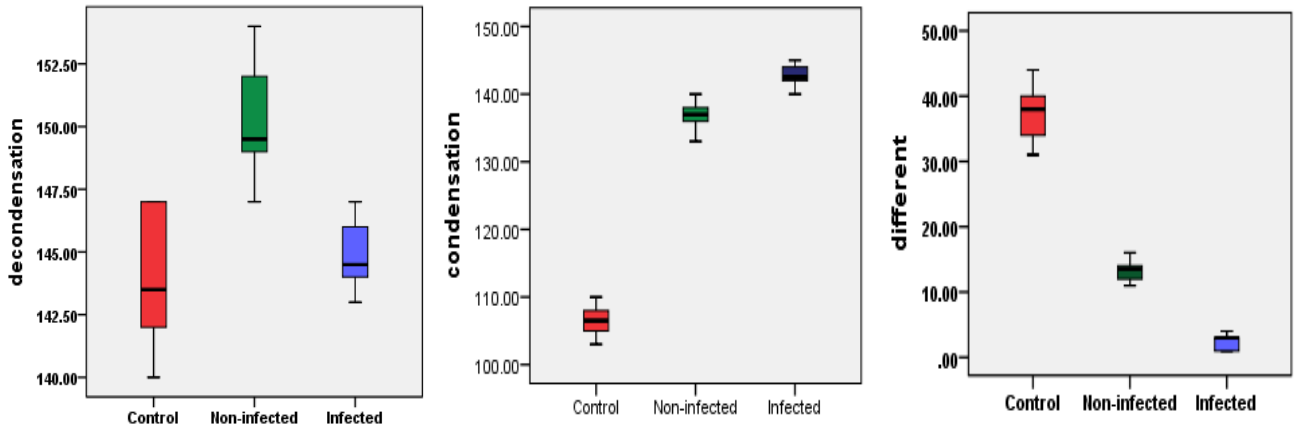


Fig. 1: Box plots of the distribution of laboratory control non-infected and infected among in the whole study of snails. In the box-plot displays, the bold line indicates the median per group, the box represents 50% of the values and horizontal lines show minimum and maximum values of the calculated non-outlier values.

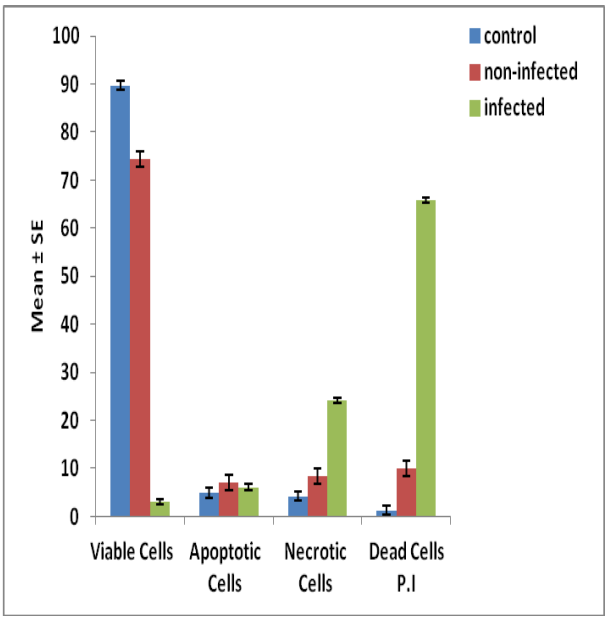


Fig. 2: Immunostaining of Annexin V in haemolymph.

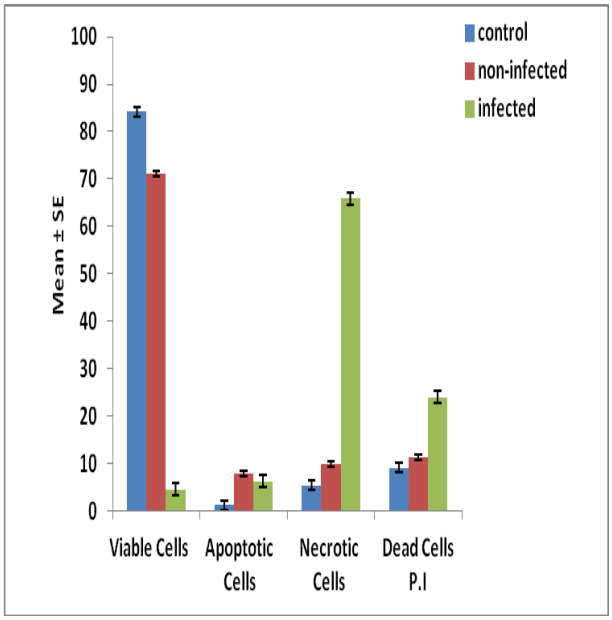
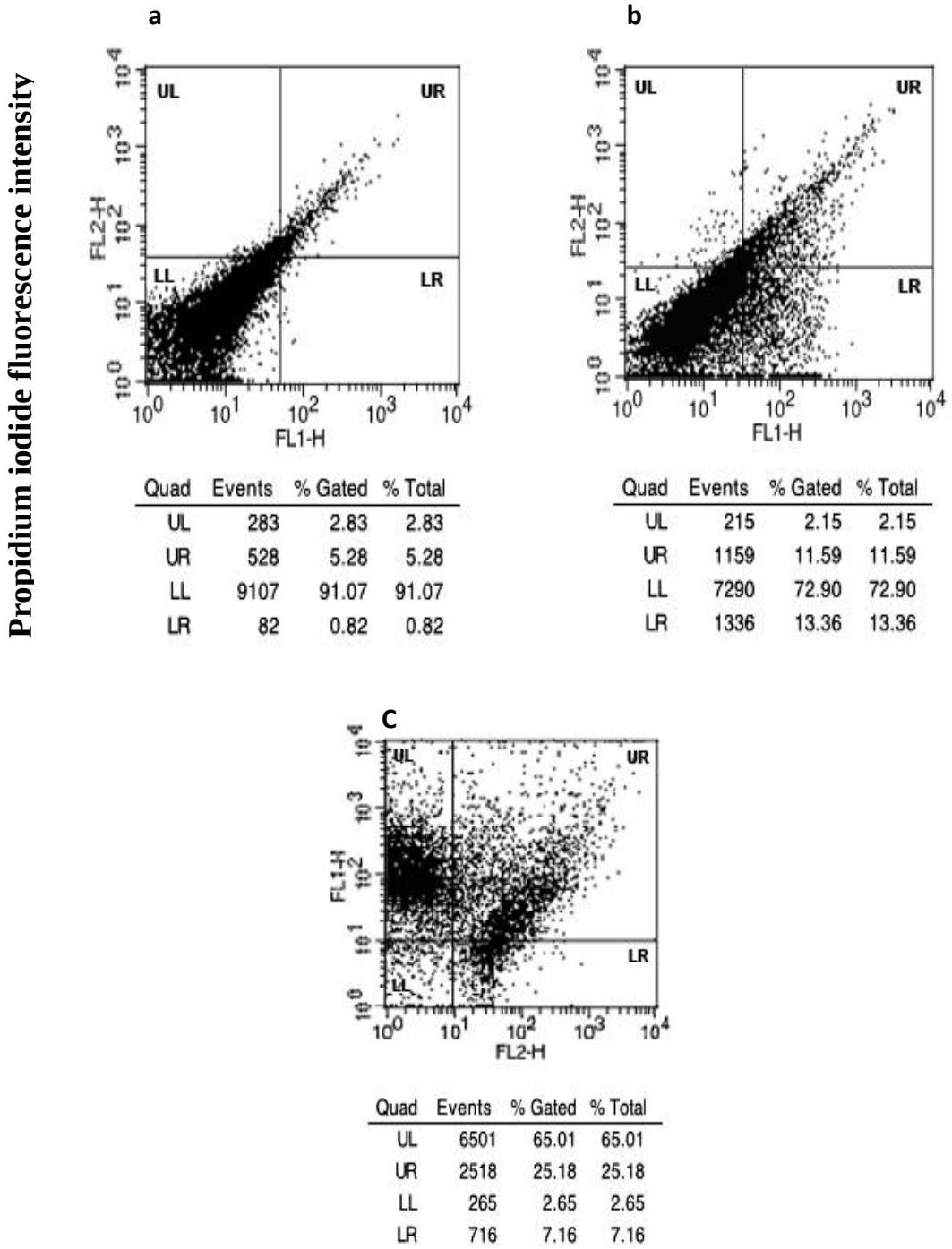


Fig 3: Immunostaining of Annexin V in tissue.



Quad	Events	% Gated	% Total
UL	283	2.83	2.83
UR	528	5.28	5.28
LL	9107	91.07	91.07
LR	82	0.82	0.82

Quad	Events	% Gated	% Total
UL	215	2.15	2.15
UR	1159	11.59	11.59
LL	7290	72.90	72.90
LR	1336	13.36	13.36

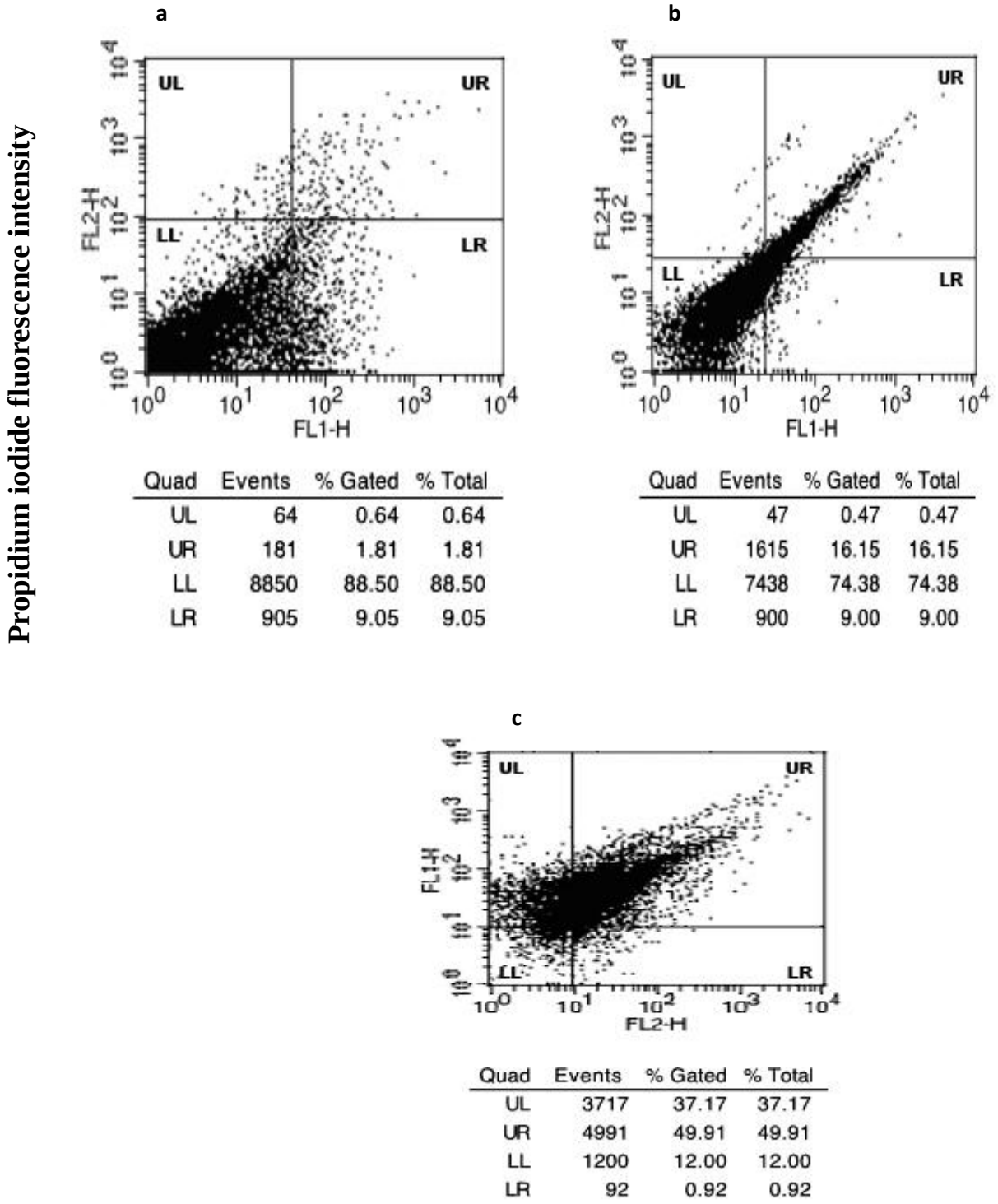
Quad	Events	% Gated	% Total
UL	6501	65.01	65.01
UR	2518	25.18	25.18
LL	265	2.65	2.65
LR	716	7.16	7.16

Fig. 4: Flow cytometric analysis of haemocytes after staining by annexin V-FITC and propidium iodide (PI): Typical dot plots of fluorescence with annexin V binding to PS on the x-axis and the distribution of haemocytes showing PI signal sets on the y-axis. The biparametric representation shows four distinct populations: (1) quadrant (Quad) LL, viable cells with no detectable dye uptake; (2) quadrant LR, apoptotic cells, which have a high annexin VFITC and low PI signal; (3) quadrant UR, the necrotic region, which exhibits the uptake of both dyes. a high annexin V-FITC and high PI signal; (4) quadrant UL, corresponding to dead cells without annexin V-FITC but with a high PI signal.

a: Immunostaining of Annexin V and propidium iodide in control haemolymph.

b: Immunostaining of Annexin V and propidium iodide in non-infected haemolymph.

c: Immunostaining of Annexin V and propidium iodide in infected haemolymph.

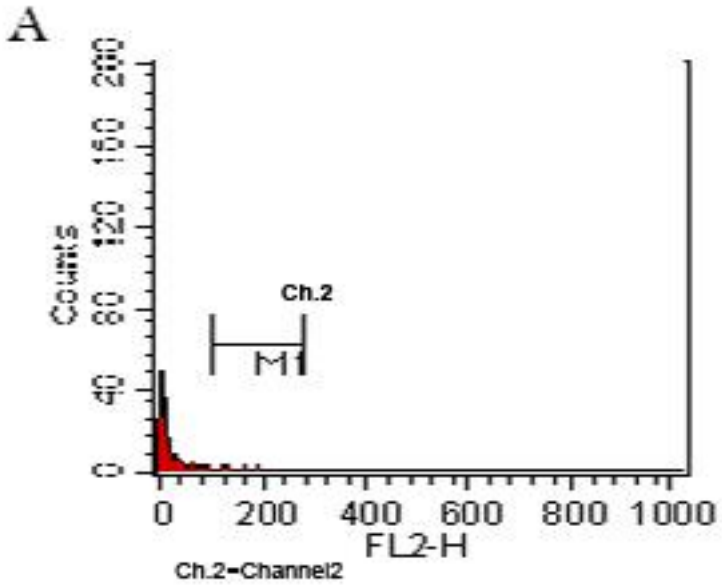


Quad	Events	% Gated	% Total
UL	64	0.64	0.64
UR	181	1.81	1.81
LL	8850	88.50	88.50
LR	905	9.05	9.05

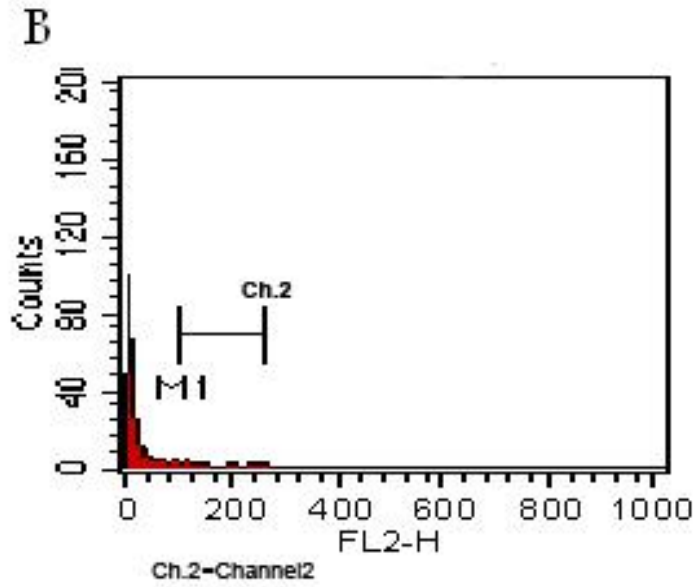
Quad	Events	% Gated	% Total
UL	47	0.47	0.47
UR	1615	16.15	16.15
LL	7438	74.38	74.38
LR	900	9.00	9.00

Quad	Events	% Gated	% Total
UL	3717	37.17	37.17
UR	4991	49.91	49.91
LL	1200	12.00	12.00
LR	92	0.92	0.92

Fig. 5: Flow cytometric analysis of haemocytes after staining by annexin V-FITC and propidium iodide (PI): light scatter of the control (a). Typical dot plots of fluorescence with annexin V binding to PS on the x-axis and the distribution of haemocytes showing PI signal sets on the y-axis. The biparametric representation shows four distinct populations: (1) quadrant (Quad) LL, viable cells with no detectable dye uptake; (2) quadrant LR, apoptotic cells, which have a high annexin VFITC and low PI signal; (3) quadrant UR, the necrotic region, which exhibits the uptake of both dyes. a high annexin V-FITC and high PI signal; (4) quadrant UL, corresponding to dead cells without annexin VFITC but with a high PI signal.
a: Immunostaining of Annexin V and propidium iodide in control tissue.
b: Immunostaining of Annexin V and propidium iodide in non-infected tissue.
c: Immunostaining of Annexin V and propidium iodide in infected tissue.

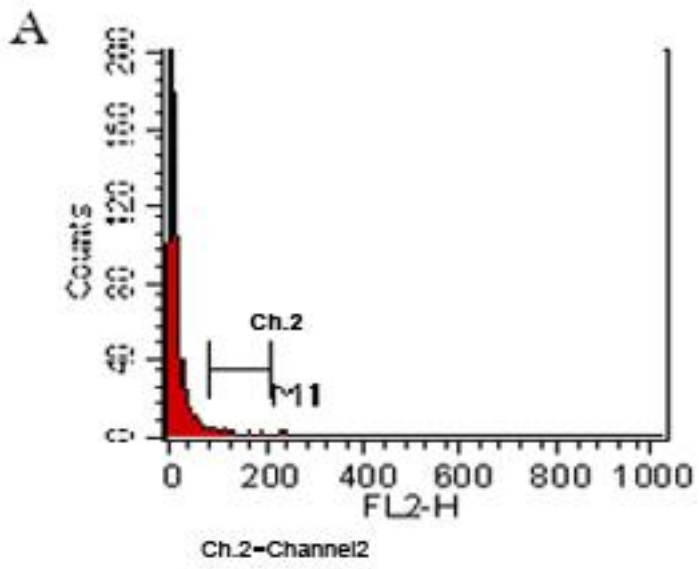


Marker	Stained Cells	Intensity of dye
All	30.38	19.44
M1	0.33	107.03

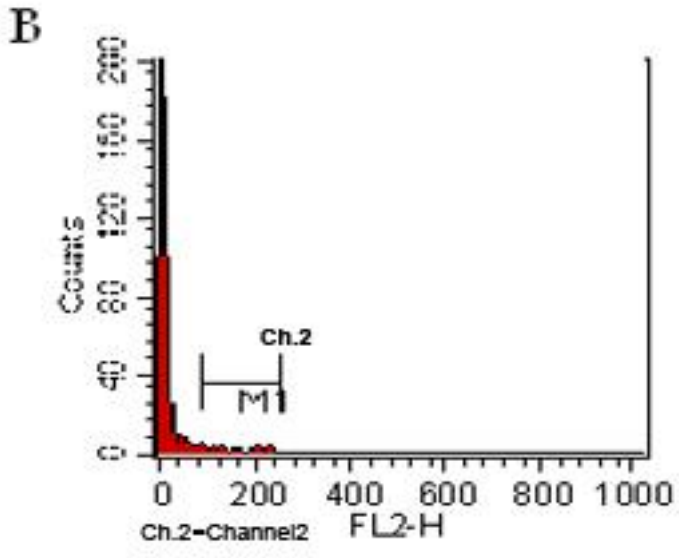


Marker	Stained Cells	Intensity of dye
All	35.36	18.95
M1	0.72	141.0

Fig. 6: DNA histogram of control group haemocyte in *Biomphalaria alexandrina* snails without exposure to miracidia: Showing that: (A) condensation mean of chromatin.
(B) de-condensation mean of chromatin.



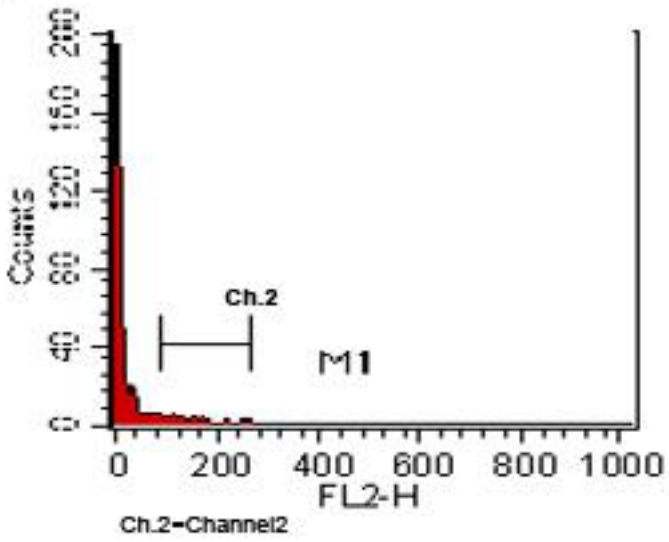
Marker	Stained Cells	Intensity of dye
All	16.75	21.78
M1	0.32	137.38



Marker	Stained Cells	Intensity of dye
All	24.31	23.05
M1	0.80	153.17

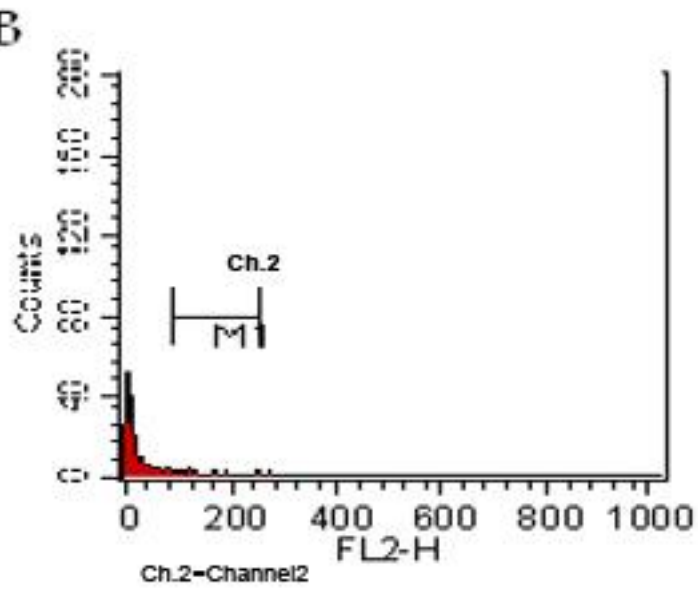
Fig.7: DNA histogram of non infected group haemocyte in *Biomphalaria alexandrina* snails exposed to *Shistosoma mansoni* miracidia showing that:(A) condensation mean of chromatin. (B) de-condensation mean of chromatin.

A



Marker	Stained Cells	Intensity of dye
All	98.77	9.59
M1	1.13	142.82

B



Marker	Stained Cells	Intensity of dye
All	4.72	23.66
M1	0.15	143.60

Fig. 8: DNA histogram of infected group haemocyte in *Biomphalaria alexandrina* snails: Showing that: (A) condensation mean of chromatin.
(B) de-condensation mean of chromatin.

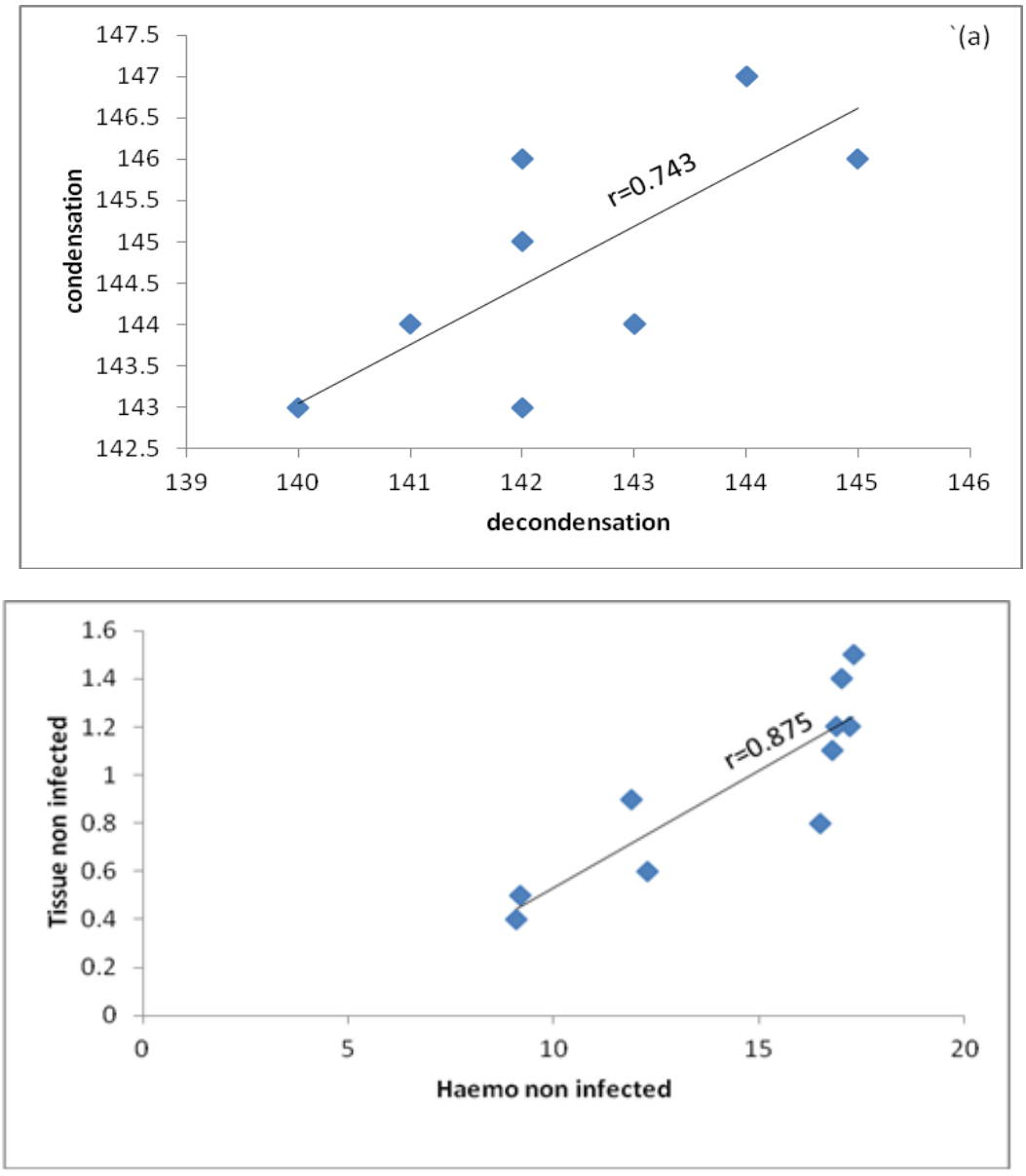


Fig. 9: Positive Pearson's correlation between (a) Haemolymph and tissue in control group (r= 0.743, $P < 0.01$); (b) Haemolymph and tissue in non-infected group (r= 0.875, $P < 0.01$)

DISCUSSION

The internal defense system (IDS) is one of the factors that influence the susceptibility pattern of the snails. This system is stimulated by the excretory- secretory products of the penetrating miracidia[15]. Its main action is mediated by the phagocytic haemocytes in cooperation with humoral components[16].

The interaction between *S. mansoni* and the IDS of the snail suggests that the haemocytes could be the main effecter element in the destruction of the parasite. The haemocytes are directly involved in the death of some encapsulated parasites[17], or in the production of soluble factors which could be cytotoxic[18]. However, the parasite can escape the IDS by two mechanisms, molecular mimicry and antigenic masking. In the molecular mimicry, the parasite expresses glycoprotein epitopes on their surfaces that mimic host molecules, while the antigenic masking is the absorption and incorporation of the snail agglutinins and haemolymph soluble components to the parasite surface[19].

A poptosis of the infected cells is thought to dampen pathogen spread yet protect the integrity of surrounding tissues by limiting

potentially damaging inflammation[20]. In the present study apoptosis was determined by flow cytometry using Annexin V (a protein known to bind phospholipids). Phosphatidylserine (PS) which normally resides on the inner membrane leaflet has been shown to be expressed on the outer surface is an early feature of apoptosis. Annexin V binds specifically to phosphatidylserine and is used as a marker of early apoptosis. Reutelingsperger and van Heerde[21] showed that each cell type has the molecular machinery to expose PS at its cell surface; this machinery is activated during the completing of apoptosis. Once PS is exposed at the cell surface it exhibits pro-coagulant and pro-inflammatory activities. Annexin V poorly bind to the PS-exposing apoptotic cell and can inhibit thereby the pro-coagulant and pro-inflammatory activities of the dying cell.

It was also reported that annexin V assay is an ideal method for measuring apoptosis and it is the only assay that could discriminate normal cells, apoptotic cells and necrotic cells[22]. The percentage of viable cells significantly increased in haemocytes of haemolymphe and tissue in non-infected group than infected group and it have been that viable cells were the most common cells that significantly increased than apoptotic, necrotic, and dead cells. The release of PS on the outer plasma membrane is considered to be one of the most important signals required for apoptotic cell recognition[23].

The data obtained in this study showed statistically significant differences in the number of viable cells infected and non infected groups of *B. alexandrine*, when exposed to the parasite. This result was in agreement with **Oliveira *et al*** [24] who found that, these differences indicated a differential behavior that may be related to the resistant or susceptible phenotype interrelated to the immunological capacity of these cells. Also **Barcante *et al*** [25] reported that the capacity of molluscs to respond strongly to stimuli depends on haemocyte viability and functional capacity. Also the increased dead cells (the most prominent cells) in infected group may be attributed to fight between miracidium and snail, resulting in death of cells. This was in agreement with **Abou-El-Naga and Radwan**[26] who reported that in susceptible (infected) snails, the haemocytes have been exposed for a long time to "non-self" and "changed self" (damage tissue caused by escaping cercariae) resulting in a higher of responsiveness of the haemocytes.

In this study, in non-infected group showed the increase of the percentage of apoptotic cells than control may be attributed to overcome the invading pathogenic organism. This was in agreement with **Sokolova**[9], a poptosis is important for the functioning of the molluscan immune system as indicated by the high baseline apoptosis rates observed in circulating and resistant haemocytes. Apoptosis also plays a role in host protection against parasites by limiting the spread of the pathogen while preventing inflammatory damage of surrounding tissues.

In infected group the increase of apoptosis may be associated with oxidative burst of haemocyte and are abolished by treatment with antioxidant suggesting that apoptosis may be induced by oxidative damage to haemocytes during killing pathogenic organism[27]. Programmed cell death with all characteristic hallmarks of apoptosis including cell shrinkage and blebbing ,chromatin condensation and DNA fragmentation and translocation of a phospholipid phosphatydilserine into the outer leaflet of the cell membrane, have described in a variety of mollusks by **Sunila and LaBanca**[28] **and Sokolova *et al***[29]. In infected group the increased of the apoptotic cells was associated with the decrease in the difference between condensations and de condensation of DNA this means failure to restore DNA. No significant change was observed in apoptotic cells present in tissue and haemolymph. There will be need for more immunological investigations to detect the mechanism of defense. Increased dead cells in infected can conclude as result of fight between pathogen and snails.

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