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Morphological Alterations in Haemocytes of Adult American Cockroach, *Periplaneta americana* (Linnaeus) (Insecta: Blattodea: Blattidae) in Response to Thermal Stress and Induced Infection

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Abstract— American cockroaches (*Periplaneta americana*) are considered as potential vectors and possible reservoirs of pathogenic organisms. They respond to any infection by mounting immune responses mediated by morphologically and functionally distinct haemocytes. To understand the effect of abiotic stress like heat, cold and biotic stress induced by infection on American cockroahes, microscopic study of haemocytes was carried out from adult *P. americana* (Linnaeus) (Insecta: Blattodea: Blattidae). In the present study, morphological alterations of haemocytes were observed at high temperature (45°C for 12 hours), low temperature (4°C for 12 hours) and following inoculation with *E. coli* bacteria compared to the control haemocytes. Stress induced necrotic changes of haemocytes like loss of cellular integrity, vacuolization, cell swelling, blebbing, margination of chromatin, membrane rupture were recorded in all the three types of stress conditions. The most affected haemocytes were Plasmatocytes, Granulocytes and Prohaemocytes. Unusual nuclear separation in a cap like bleb formation and margination were observed at low temperature. Cellular aggregation and phagocytic extensions of Granulocytes and Plasmatocytes were visible after induced infection. This is the first study where the changes in the primitive immune cells of American cockroaches have been reported in response to abiotic and biotic stress. Hemocyte structural modifications and changes observed in the present study have potential implications in the study of stress response in *P. americana* in the extreme environment and in destruction of pathogens. Cellular aggregation and phagocytosis are characteristics defence reactions which are not found in thermal stressed cells. Although vacuolization, blebbing and chromatin margination were common alterations in response to all types of stresses applied in this study.

Keywords— *Haemocyte, Morphology, Thermal stress, Infection, Cockroach*

I. INTRODUCTION

Insect immune system is mainly composed of cellular and humoral responses. Haemolymph of insects possess a variety of cells called haemocytes which perform many vital functions such as nodule formation, phagocytosis, encystations [1], [2], [3]. Other than combating different infections hemocytes are also involved in nutrient and hormone transport, protein storage and wound healing [4]. Hemocytes do not show any clonal selection mechanism and memory cell production. Insect haemocytes are a combination of a variety of cell types involved in mounting defence reactions against non self components. Though there is considerable variation in morphology and development pattern in different insect species [5] most of the cell types are similar functionally [6].

On the basis of shape, size and staining reaction and nuclear character hemocytes of different insect orders were characterized [1], [2], [3], [7]. Based on morphology and function there are several types of hemocytes described in insects of order Blattodea. Cockroaches belonging to order Blattodea are an ancient group of insects able to tolerate a wide range of environmental fluctuation [8], [9]. It is an omnivorous species and major pest of food and stored products. They also act as an important vector of the number of pathogenic agents [9].

Jones (1962) [2] described two types of hemocytes viz. Plasmatocytes and Cystocytes in *Periplaneta americana*. Three other types of hemocytes viz. Prohemocytes, Granulocytes and Spherulocytes were reported by Boerwald (1975) in the same insect [10]. Insect growth and development is influenced by different environmental factors especially by fluctuating or extreme temperatures, crowding, starvation, poison, injury and pathogenic infection [1]. As insects are poikilothermic, increase or decrease of temperature causes stress in the insect body and affects its metabolism [11], [12]. However, different insect species have their own range of temperature tolerance for proper growth and development [13]. On the other hand, several studies on *P. americana* reported that the insect has cold receptors and becomes immobile when kept in freezing temperature [14, 15]. Conversely, *P. japonica*, Karny, is able to survive below freezing temperature [16]. But very little information is available on

the morphological aberration and effect of stress on the hemocytes of *P. americana*. Hence, the present study aims at understanding the changes of cellular morphology in adult *P. americana* during thermal stress and induced microbial infection. This is the first report on the abiotic and biotic stress induced cellular necrosis in adult *P. americana*.

This manuscript is organized as follows, Section I contains the background and focus of the study, Section II contains the related work on the problem done by other researchers, Section III contains description of specific methods used for the present work done, Section IV describes results and discussion and Section V concludes research work with future directions.

II. RELATED WORK

[17] Study on tropical tasar silkworm larva *Antheraea mylitta* showed that temperature has a great impact on the morphology of the haemocytes. They identified six types of haemocytes in the economically important *A. mylitta*. Differential Hemocyte counts (DHC) were varied from the normal count when the ambient temperature were changed to either chilling (40^0C) or heating (50^0C) to cause temperature stress. It was reported that Prohaemocytes, Plasmatocytes and Granulocytes are the most affected cellular types in these insects under exposure at 50^0C temperature for one hour. Cellular alterations in the form of vacuolization, nuclear fragmentation, loss of cytoplasmic compactness and even death were observed.

[18] According to Ryan and Nicholas (1972) when foreign particles were injected into the haemocoelomic space of adult *Periplaneta americana*, immediate phagocytosis was observed. These cellular responses were accompanied by variation in number of circulating hemocytes as well as proportion of populating hemocytes.

[19] 4t th instar larvae of *Agrotis epsilon* were treated with *Bacillus thuringiensis* and farnesol and cellular abnormalities were noted under Scanning Electron microscope. The insecticidal activity of the bacteria and farnesol showed both time and dose depentent effect. They reported plasmatocyte membrane with thin pseudopodia rose from the cell surface.Also Prohemocytes abnormal surface raising into concentric circles were observed.They showed numerous structural changes like curling, shrinkage, swelling and vacuolation of treated cells. Due to the effect of bacterial toxins Granulocytes elongated into long extended filaments.

[20] Kiuchi *et al* investigated the heat induced changes on plasmatocytes and granulocytes.They correlated insect growth with increase in temperature.

Thus, based on the previous studies, it seems that cellular alterations are an important stress adaptive response in insects.

III. METHODOLOGY

Sampling, rearing, collection of hemolymph and smear preparation

Adult cockroaches (*Periplaneta americana*) were collected from residential areas and are reared in a box. They were fed biscuits and kept in 12 hours light and dark environment in room temperature (approx. 27 °C) for a week. For collection of hemolymph, cockroaches were immobilized by lukewarm water and hind pairs of legs were cut using fine scissors sterilized in 70 % ethanol. Collected hemolymph was placed on a slide and a smear was prepared.

Identification of hemocytes using light microscopy

Uniformly smeared hemolymph preparation on glass slides were kept for drying. Smears were fixed in methanol for 5 minutes and stained with Giemsa stain (9:1 dilution) following the standard protocol [21]. Permanent slides containing blood cells were identified and photographed using Carl Zeiss Axio Scope A1microscope.

Application of stress to observe the morphological changes of haemocytes

Thermal Stress

Adult cockroaches were kept in a container with food and exposed to a heating temperature of 45°C for a 12 hours period in an incubator. The temperature of the incubator was increased gradually from 27 °C to 45°C. Again, a separate group of adult cockroaches were exposed to a chilling temperature of 4°C for 12 hours in a refrigerator. Control insects were kept at room temperature (27 °C) for 12 h period.

Infection of cockroaches with bacteria to induce stress

Insects were infected with the *E. coli* bacteria. The bacteria were cultured in Luria Broth medium overnight at 37ºC. Bacterial mass was collected by centrifugation and the pellet was suspended in physiological saline to a concentration of Optical Density value 0.5 (520 nm) (final concentration of bacteria reaches approximately 290 $x10^6$ Colony Forming Unit / ml). About 20 µl of this diluted bacterial suspension was then injected to the adult insect's body cavity using a micro syringe. After 24 hrs haemolymph were collected for cytological observations.

Light microscopic observation

Haemolymph were collected from the stressed insects separately, smear prepared and stained with Giemsa (Merck). Cellular abnormalities were visualized using the light microscope and photographed under high power magnification.

IV. RESULTS AND DISCUSSION

Normal haemocyte profile found in adult *P. americana*

Light microscopic observation of prepared smear from adult *P. americana* haemolymph showed five different haemocyte types i.e. Prohaemocytes, Plasmatocytes, Granulocytes, Oenocytoids and Adipohaemocytes.

Description of cells

Prohaemocyte (PR)

These were round or oval cells (about 1.4 to 2.8 µm in diameter), relatively large, almost centrally located prominent nucleus. A few cytoplasmic granules present. Among all the haemocytes obtained these cells were the smallest one (Figure1a).

Plasmatocyte (PL)

Cells were larger than PR, variable in shape ovoid, spindle or amoeboid (2.8 to 5.6 µm long and 1.4 to 2.1 µm wide). Nucleus occupies about 40% of the cell volume. Cytoplasm was finely granular (Figure1b, 1d).

Granulocyte (GR)

Large cells of variable size (3.2 to 5.5 µm long and 2.1 to 4.2 µm wide). Cytoplasm packed with granules (Figue 1c).

Adipohaemocyte (AD)

Irregular shaped large cell. Numerous lipid filled pouches were found in the cytoplasm (Figure1e)

Oenocytoid (OE)

Large round or oval cells (3.3 to 4.5 µm diameter). Size of the nucleus was relatively small. A few cytoplasmic granules were present (Figure1f).

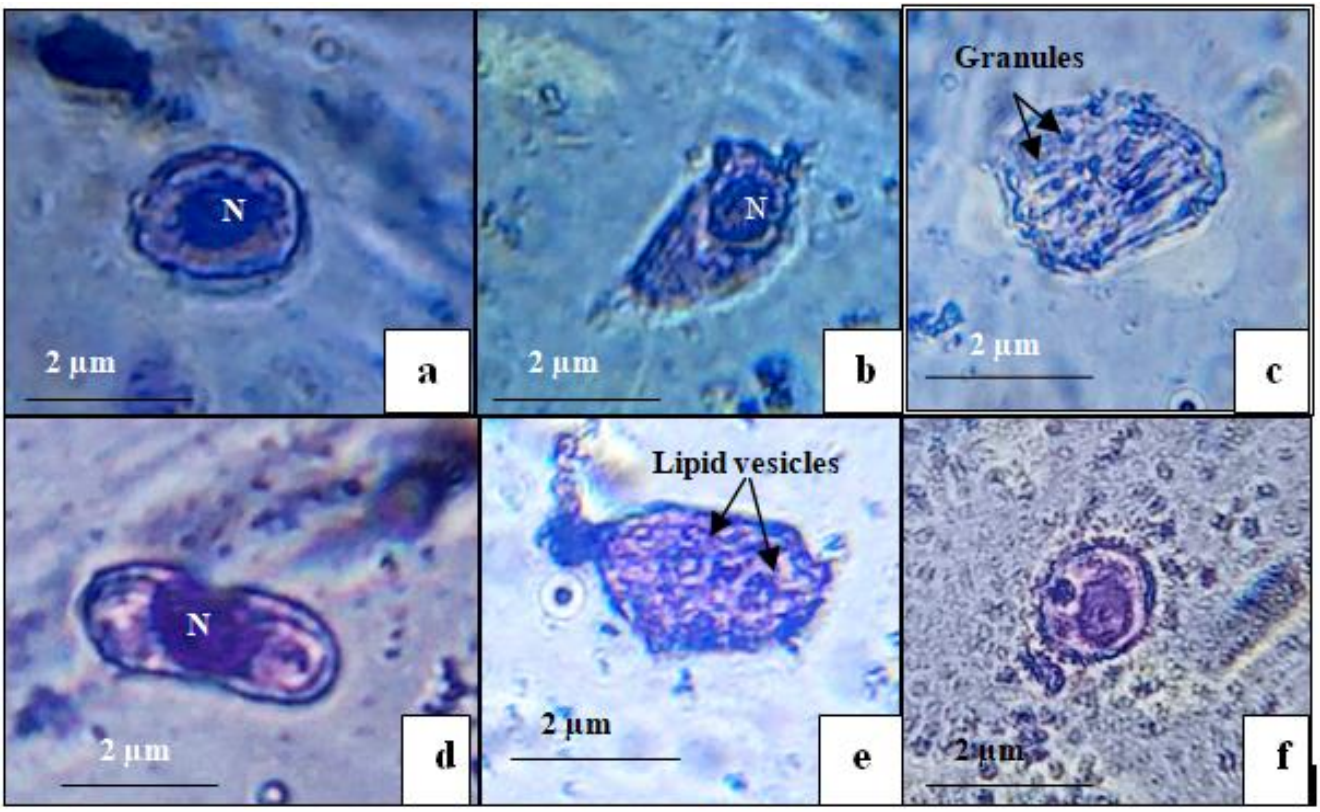

Figure 1. Photomicrographs of haemocytes of *P. americana*. **a.** Prohaemocyte, **b.** Plasmatocyte, **c.** Granulocyte , **d.** Plasmatocyte, **e.** Adipohaemocyte , **f.** Oenocytoid [400 x magnification]

Impact of heat stress on cellular morphology

Heat stress had a pronounced impact on the shape and contour of the haemocytes. PR which was the small, immature stem cells showed vacuolization and loss of cellular integrity. In some PR membrane wrinkling was found (Figure 2a).

PL and GR were found as the most abundant haemocytes in control specimens and phagocytic PL were pleomorphic with a variety of shape and sizes. In high temperature, PL developed numerous vacuoles in the cytoplasm and cell swelling, membrane blebbing and rupture were noted (Figure 2b).

Control GR was found in irregular shape with small and large granules in the cytoplasm. The Nucleus was small and acidophilic cytoplasm was clearly visible. But heat induced GR showed karyolysis and margination of chromatin material (Figure 2c and 2d).

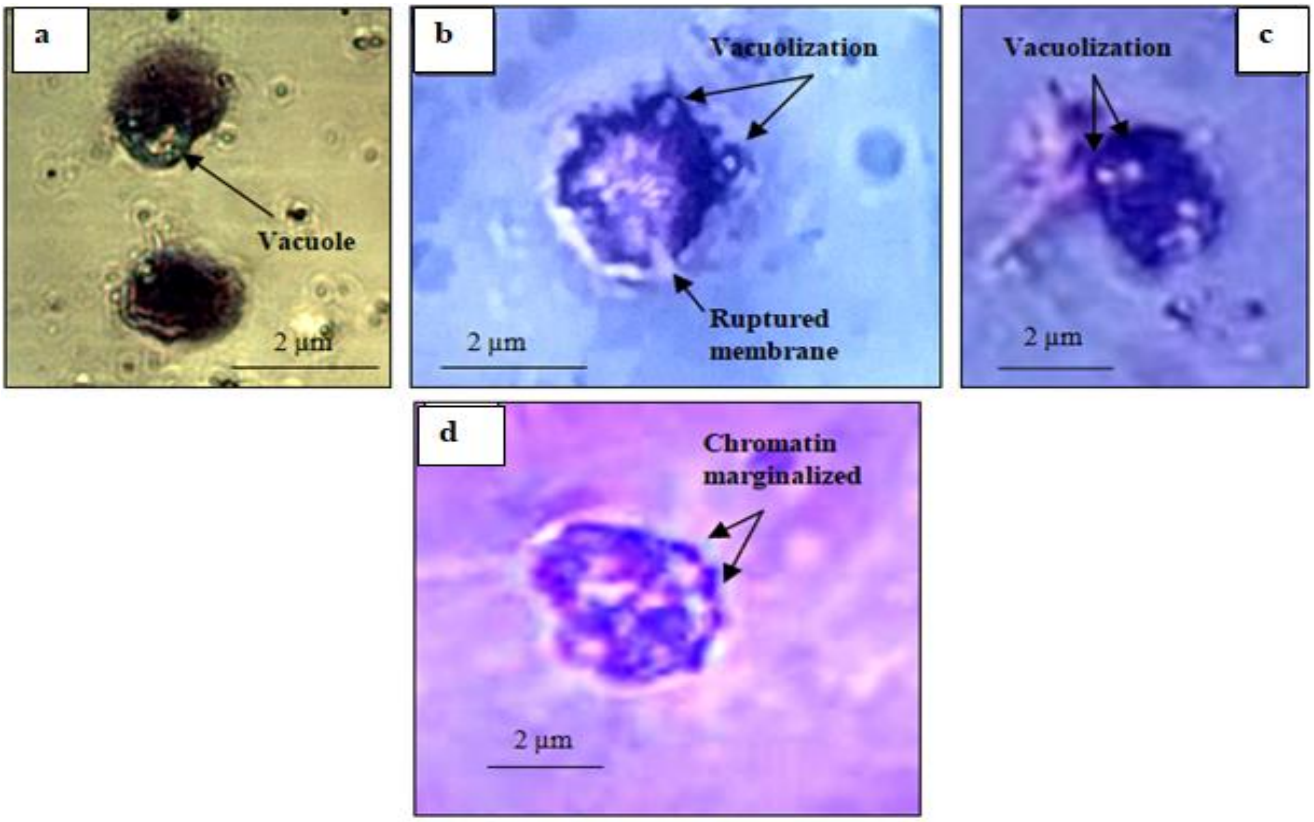

Figure 2. Effect of heat stress on **a.** Prohaemocyte **b.** Plasmatocyte **c.** Granulocyte with vacuoles **d.** Granulocyte with marginalized chromatin [400 x magnifications]

Impact of cold stress on cellular morphology

After the cold shock cockroach haemocytes were accompanied by similar necrotic changes as the heat stress. PR developed common necrotic features like membrane chromatin margination and nuclear disintegration (Figure 3a and 3c). GR showed multiple blebbing of the plasma membrane and cellular disintegration were seen (Figure 3b). Blebs are bubble-like protrusion from the cell membrane that develops as a result of cytoskeletal contraction.

In some cold stressed insects an unusual cellular alteration was observed in PR and PL. Figure 4a clearly represented many Giemsa stained cells with these altered structures. In these cells, the whole nuclear material is marginalized in one side like a cap deforming the shape of the haemocytes (Figure 4b). Cold stress also induced aggregation of haemocytes.

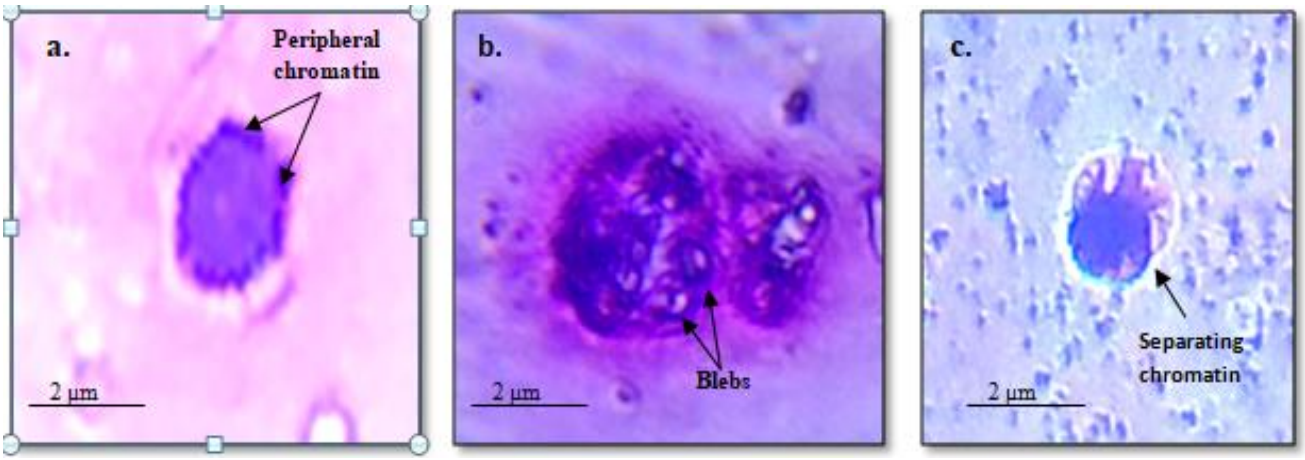

Figure 3. Effect of cold stress on a**.** Prohaemocyte b. Granulocyte c. Prohaemocytes with nuclear disintegration [400 x magnifications]

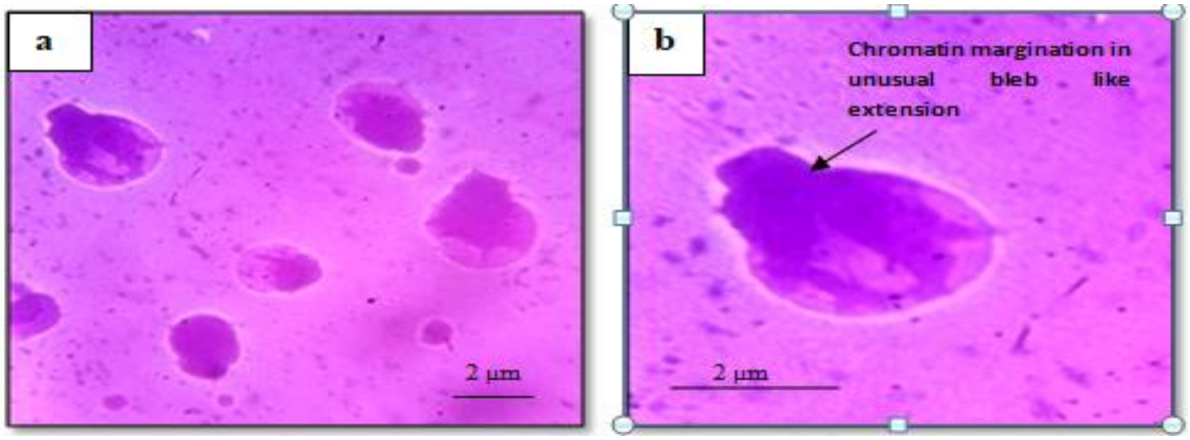

Figure 4. Morphological alteration due to cold stress in prohaemocytes showing unusual nuclear margination and cap like bleb formation in a. (40 x magnifications) and b. (1000 x magnifications)

Impact of induced infection on cellular morphology

Considerable morphological alterations and cellular abnormalities were found in adult *P. americana* after treatment with *E. coli* bacterial infection. GR were found with phagocytic extensions shown in Figure 5a as proof of phagocytic activity.

After the microbial infection, normal PR with compact membrane and large nucleus changed into a swollen cell with abnormal shape. Cell cytoplasm of the haemocytes also showed vacuole formation (5 b) and nuclear materials disintegrated and accumulated marginally (Figure 5 b, c and d).

Most frequently observed haemocytes like PL and GR formed aggregates that probably help in cellular encapsulation. The first step in cellular encapsulation is the contact of immune cells with the invading pathogen and then releasing the cellular content by degranulation which is perfectly done by the GR. Formation of cellular aggregates by GR is evident from Figure 5 c. Size of PL and PR became enlarged and often found with abnormal shapes. In Figure 5 d PR was shown in a comma shaped structure.

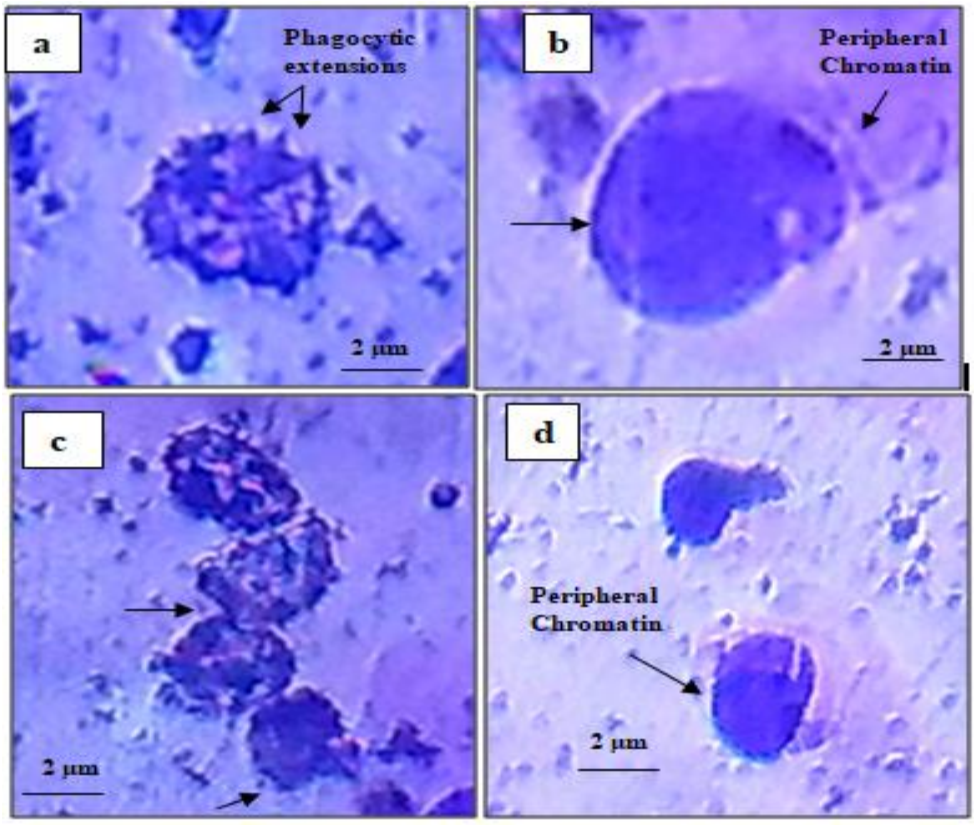

Figure 5. Morphological alteration of haemocytes due to *E. coli* infection in *P. americana*. a**.** Granulocytes with phagocytic extensions, b**.** Plasmatocytes is swollen and enlarged with vacuolated cytoplasm and peripheral chromatinization. c. Granulocytes forming aggregates. d. Prohaemocytes with altered cell shape. Arrow indicates marginal chromatin condensation (in Figure c and d).

Discussion

In the present study five morphotypes of haemocyte i.e. PR, PL, GR, OE and AD were obtained from untreated adult *Periplaneta americana*. In a separate study Arnold (1972 a) reported cystocyte [22] and Boerwald (1975) [10] reported spherulocyte in *P. americana* which we did not find in our prepared smear. Consistent with the results of the previous studies PR was obtained as a small, rounded cell whereas size and shape of the PL and GR varied.

When insects were incubated at 45°C for 12 hours, haemocytes showed extensive vacuolization, membrane blebbing and even rupture of cell membrane but PL and GR were not identified with any phagocytic extensions. Similar impact of heat stress was observed in the larva of *Antheraea mylitta* at 50^0C temperature for one hour [17].

During cold stress the cellular abnormalities and necrotic changes recorded are almost in accordance to alterations found in heat stress. In some specimen peculiar nuclear margination with cap-like bleb formation were noted in PL and PR. Compared to heat stress, cellular damage was more extensive after cold shock. According to Bradt et al. (2018) adult American cockroaches cannot survive several days at $\leq 10^0$c which confirms the chilling effect on the insect [23].

E. coli injected *P. americana* haemocytes presented identical cellular changes as thermal stress. In addition to this, filopodia formation and phagocytic extensions were observed in PL and GR. In study of Scott (1971) active uptake of sheep and chicken erythrocytes on the surface of *Periplaneta americana* phagocytes *in vitro* was demonstrated [24]. Larvae of *Agrotis epsilon* when treated with *Bacillus thuringiensis*, cellular abnormalities like extensive vacuolization of the cytoplasm of PLs and GRs, membrane damage and cell lysis were observed which is also evident in the present study after microbial injection [19]. GR were found to form aggregates suggesting successive encapsulation of bacteria.

V. Conclusion and Future Scope

This is the first study where the morphological changes in the most ancient defence cells of American cockroaches have been reported in response to differences in temperature and induced infection. Extreme fluctuations of temperature and pathogen infection can affect the hemocytes morphology of American cockroach is evident in the present study. These stresses induced common cellular modifications like cell swelling, vacuolization, bleb formation and chromatin margination in PL, GR and PR. In response to cold stress unique nuclear cap formation were noticed only in PR and PL. GR aggregation and phagocytic extension were exclusively observed after pathogen introduction. However, further investigation on the effect of various biotic and abiotic stresses on the immune system of *P. americana* in different developmental stages may need to be performed to understand the effect of environmental factors on the immune system of this pest species. However, the response of hemocytes against other pathogens like fungi, protozoans and helminths needs to be elucidated. Ultrastructural analysis can be applied to study detail morphological changes with stringent specimen preparation. There are several controversies regarding classification and identification of hemocytes [5]. Monoclonal antibodies can become a promising tool for identification of specific hemocytes [25].

LIST OF ABBREVIATIONS

CFU: Colony Forming Unit; PR: Prohaemocyte; PL: Plasmatocyte; GR: Granulocyte; OE: Oenocytoid; AD: Adipohemocyte; OD: Optical Density

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