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Investigating the haemocyte - mediated immune response in American cockroach (*Periplaneta americana*) to *Escherichia coli* infection

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Abstract

The immune system of the American cockroach (Periplaneta americana) is primarily innate, relying on various cellular and humoral responses to defend against pathogens. Haemocytes play a central role in the cockroach's immune system, acting as the primary cells involved in defence mechanisms such as phagocytosis, encapsulation, and the production of immune molecules. In the present study, the impact of bacterial infection on haemocyte-mediated immune responses in Periplaneta americana was studied. For the experiment, laboratory-cultured Gram-negative bacteria, Escherichia coli, were injected into the abdominal cavity of the insect. Haemolymph was collected 2 hours, 5 hours, and 24 hours post-infection from the treated and control adult cockroaches, and morphological changes were noted. Total haemocyte counts (THC) and differential haemocyte counts (DHC) were also compared between control and inoculated host specimens. THC levels rose at 2 hours of infection, reached a significant peak at 5 hours of infection, and declined at 24 hours of infection. A significant decline in the number of prohaemocytes and an increase in granulocytes were found throughout the infection period. Among all the haemocytes, granulocytes were identified as the major defense cell, presenting an elevated cell count and being associated with aggregation at the site of infection, vacuolation related to phagocytosis, and also being involved in the formation of thin, finger-like extracellular traps.

Keywords: *E.coli*, granulocyte, haemocytes, infection, *Periplaneta Americana*.

Introduction

With some 4000 species in 460 genera, cockroaches are among the oldest winged insects known. The American cockroach, *Periplaneta americana* (Linneaus, 1785) in urban areas acts as a major pests and potential disease transmitter[1,2]. According to Bell and Adiyodi cockroaches belong to the class Insecta, order Blattodea and Family Blatellidae[3]. Zurek and Schal observed that these insects can attach bacterial spores, protozoan cysts, and helminth eggs in specially adapted mouth parts, legs, wings, and other body parts from polluted and unhygienic habitats during foraging[4]. Cockroaches are considered one of the most adapted insects on earth. They can survive extreme, harsh environmental conditions, are least affected by pathogens and pollutants, and at the same time have high reproductive potential.

The most important domestic species of cockroach is the American cockroach. In the urban environment, *P. americana* occurs outdoors and indoors in a wide range of habitats. The close association of this insect with humans exposes them to many allergens. Presence of a diverse and complex microbial community in the hindgut of *Periplaneta americana* was reported by Tinker, K.A., Ottesen, E.A.[5]. Many pathogenic bacteria like *Escherichia coli*, *Salmonella* spp., *Shigella*, coliforms, and *Staphylococcus aureus* were isolated from the guts of cockroaches, which they regularly shed in their fecal matter[6-8]. Rust et al. expressed concern on the extensive use of synthetic chemical insecticides in households and in different public places like hospitals, hotels, and public transport to restrict and control the prolific breeding of this particular pest[9]. According to Chang et al. these chemical insecticides lead to insecticide resistance in the insect population and impart deleterious effects on the environment and human health[10]. To control this insect pest, a biological control method may serve as the best alternative approach. Understanding the cockroach immune system could lead to the development of pathogen-based control strategies that reduce the need for chemical pesticides.

Insect blood is composed of haemocytes and plasma. Jones observed that insect cellular defense is mainly contributed by plasmatocytes and granulocytes[11]. Insect haemolymph cells and fat bodies synthesize antimicrobial peptides when microbial infection is induced[12-14]. *Periplaneta americana* was found to produce lysozyme-like inducible antibacterial peptides[15-16] and Eleftherianos et al. found that there is a complex genetic cascade in insects for controlling bacterial infection[17]. Ryan and Karp stated that soluble protein antigens of *Enterobacter cloacae* can induce a humoral immune response, which gives *P. americana* a long-term immunologic memory[18]. Basseri et al. injected Gram-positive *Micrococcus luteus* and Gram negative

Escherichia coli into the abdominal cavity of an American cockroach and purified antibacterial protein[19].
In this context, the aim of the present investigation is to assess the effect of a Gram-negative, laboratory strain of *Escherichia coli* infection on the haemocyte-mediated immune response of *Periplaneta americana.* To identify the haemocytes associated with cell mediated immunity against invading pathogens and determine the immune response dynamics based on changes in haemocyte number and immune response intensity.

Material and Methods

Collection and rearing of the cockroach, *Periplaneta americana*: Cockroaches were collected from the kitchen and market areas of Dum Dum from August 2023 to March 2024. Live cockroaches were reared in the laboratory of Barasat Government College in a plastic container at 75 to 86% relative humidity (rh) under a natural photoperiod (12 L: 12 D) cycle and room temperature (29 ± 3℃). They were fed with finely ground biscuits, and water-soaked small pieces of sponge were provided as a drinking water source.

Bacterial inoculation in *P. americana* and haemolymph collection: The bacterial strain used in the study is a Gram-negative laboratory strain of *Escherichia coli,* kindly provided by the Department of Biochemistry, University of Calcutta, West Bengal, India. This bacterial strain is non-pathogenic to humans. Prior to bacterial inoculation, cockroaches were separated into two subgroups each; one subgroup was the treated group, and the other was the control group. For inoculation, a cuticle perforation was made in each insect, between the second and third segments of the abdomen, and 20 µl of bacterial suspension in PBS (containing 3.6×10^6 cells/ml) was injected aseptically into each insect of the treated group, whereas 20µl of sterile distilled water was injected into the insects of the control group following the method of Bell et al.[20]. As the possible time span required for the production of antimicrobial peptide is 5–12 hours, 20µl of haemolymph were extracted two hours, five hours, and twenty-four hours after inoculation using a microsyringe, and THC and DHC were estimated. Haemolymph is obtained from adult cockroaches by severing antennae and blood was collected directly on slide as adopted by Landois and Landois[21], Patton and Cragg[22], Auber[23].

Differential haemocyte count (DHC): DHC of untreated (control) and pathogen-treated cockroaches was done. Haemolymph collected from the severing of one antenna was taken on a slide, and a thin blood smear was prepared. The smear was fixed with 100% methanol following Arnold and Hinks[24]. The smears were stained with a 3% Giemsa stain. A minimum of 200 cells were counted sequentially per smear under a compound microscope at a magnification of 400x[25,26]. Haemocyte morphology and differential counts of control insects were taken. Morphological alterations and differential count changes were noted in treated insects in response to infection. Photographs were taken with an Olympus CH20i trinocular microscope. The percentage of different cell types was calculated. The data was subjected to statistical analysis using the Student's t-test.

Total Haemocyte Count (THC): Before the start of the experiment, a haemocytometer was cleaned with double-distilled water, wiped with tissue paper, and covered with a coverslip. Using a sterile scissor, the tip of the antennae was cut, and haemolymph was directly collected in the slide. 15 µl of Giemsa stain and an insect saline mixture were mixed with the haemolymph present on the slide. Next, the prepared solution was taken into the cleaned haemocytometer, and counting was done. The pipette tip was carefully introduced in the space between the coverslip and haemocytometer, and the solution moved inside the chamber through capillary action. Cells in the four corners and central squares of the haemocytometer were calculated. The number of haemocytes per cubic millimeter was calculated with the following formula proposed by Jones[11]:

$$\frac{\text{Haemocytes in five 1mm squares} \times \text{dilution} \times \text{depth factor of chamber}}{\text{Number of squares counted}}$$

Cell measurements: Haemocyte cell measurements were taken using ocular and stage micrometers, and mean and SD values were calculated.

Statistical analysis: A statistical comparison of *P. americana* THC and DHC counts between the control and treated groups was done using a paired Student's t-test ($p < 0.05$) using Microsoft Excel. The values were presented as mean ± SD, and the difference between haemocyte counts at different hours after treatment was also analyzed statistically.

Results and Discussion

Morphological features of individual haemocytes isolated from the control cockroach: Three well defined cell types were observed under a microscope in all the blood smears studied. These were prohaemocytes, plasmatocytes, and granulocytes. Prohaemocytes are commonly recognized as small (average size: 20.03 ± 2.24 µm) spherical cells. The nucleus covers most of the cytoplasm. The plasma membrane was smooth and even (Figure-1a). Plasmatocytes were identified as large, ovoid cells (average length 32.95 ± 4.82 µm and width 26.61 ± 4.55) with clear cytoplasm and a centrally located nucleus (Figure-1b). Cells with an irregular shape and uneven membrane, many cytoplasmic granules, and a relatively small central nucleus were the characteristics utilized for granulocyte identification (Figure-1c). Granulocyte size varied from 32.25 to 57.16 µm, and width ranged from 27.64 to 42.87 µm. The morphology of the other three specialized haemocytes was noted. These were oenocytoid, spherule cells, and adipohaemocytes. Oenocytoids are very large cells (average length 52.88 ± 11.03µm and width 40.88 ± 11.13µm) with a lobulated nucleus but clear cytoplasm (Figure-1d). Spherule

cells are one kind of granulocyte with large granular inclusions (Figure-1e), and adipohaemocytes were irregular shaped cells filled with lipid or fat droplets and other granular material (Figure-1f), (Table-1).

Table-1: Haemocyte size measurements using micrometer of control *P. Americana*.

Type of Haemocyte	Size (µm)	
	Length (Mean ± SD)	Width (Mean ± SD)
Prohaemocyte	14.29 - 21.43 (20.03 ± 2.24)	14.29 - 21.43 (20.03 ± 2.24)
Plasmatocyte	27.64 - 42.87 (32.95 ± 4.82)	18.43 – 28.58 (26.61 ± 4.55)
Granulocyte	32.25 – 57.16 (40.94 ± 8.70)	27.64 – 42.87 (31.80 ± 4.82)
Oenocytoid	42.87 – 69.12 (52.88 ± 11.03)	28.58 – 55.29 (40.88 ± 11.13)
Adipohaemocyte	35.72 – 71.45 (56.44 ± 11.71)	28.58 – 57.16 (38.58 ± 12.86)
Spherule cell	27.6 – 36.9 (32.7 ± 3.83)	23.04 – 32.25 (26.72 ± 3.44)

Sample size n=10

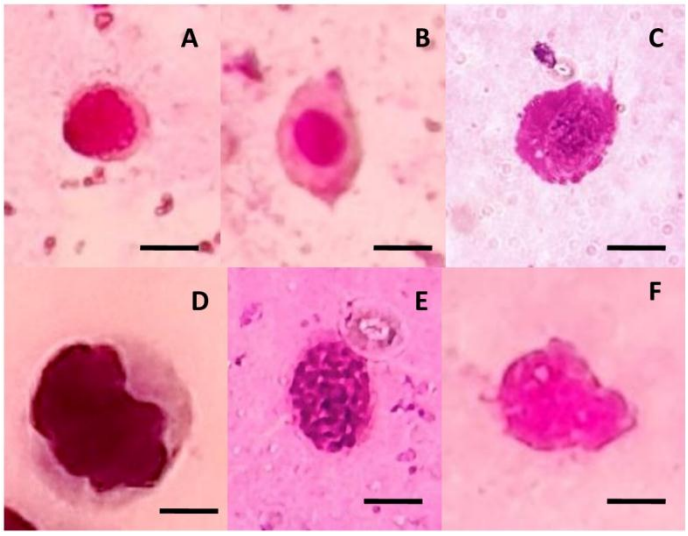

Figure-1: Giemsa stained haemocytes from *Periplaneta americana*. a. Prohaemocytes, b. Plasmatocytes, c. Granulocytes, d. Oenocytoid, e. Spherule cell, f. Adipohaemocyte. Line bar 15 µm.

At different hours post injection of the laboratory strain of *Escherichia coli* in adult *P. americana*, it was observed that the THC count of treated insects varied from the control group. Table-2 shows that at 2 hours post-inoculation, the THC value in the control specimen is lower compared to the treated group, though it is not statistically significant. But 5 hours after treatment, the THC count was significantly higher in the E. *coli*-treated sample. The THC value decreased in the bacteria-treated specimen after 24 hours post-inoculation.

24 hours post-injection, blood smears showed different kinds of cellular responses. The most significant changes were observed in granulocytes. These cells were found to form distinct filopodial extensions (Figure-2a). Numerous granulocytes cytoplasm were distinguished to form variously sized membrane-bound vacuoles (Figure-2b). Other forms of altered behavior of haemocytes noted were aggregation (Figure-2c) and blebbing of the granulocyte membrane (Figure-2d). These indicate granulocytes becoming necrotic. The results of the effects of *E. coli* injection on the DHC of adult *P. americana* haemocytes are presented in Table-3. It was found that two hours after infection, the prohaemocyte count significantly decreased from the control specimens, but no significant difference was found in the plasmatocyte count, only a slight decline in number was noted. Granulocytes increased in number from control but were not statistically significant. In five hours post-infection, both prohaemocyte and granulocyte counts changed significantly, showing an increasing pattern in the gametocytes and a decreasing level for prohaemocytes. Plasmatocytes again did not show much deviation from the control result. At 24 hours post-infection, prohaemocyte values showed a significant decline as before, but granulocytes manifested a recovery pattern in the result. The elevated granulocyte level was not statistically significant. Plasmatocyte counts increased compared to previous hours observation, but the value was statistically non significant from control cell counts (Figure-3a, b and c). Cockroaches inoculated with *E. coli* showed 80% mortality after 48 hours of infection.

Table-2: Total hemocyte count (THC) in control and treated *P. americana*

Hours post-inoculation	THC	
	Control	Treated
2hours	$10240 \pm 123.59^{a.1}$	$12660 \pm 516.13^{a.1}$
5 hours	$10,000 \pm 131.20^{a.1}$	$22880 \pm 495.58^{c.2}$
24 hours	$10040 \pm 109.35^{a.1}$	$13267 \pm 161.10^{b.1}$

Values are expressed as Mean ± SD (n = 3). Mean ± SD with the same letter in a row are not significantly different at $p < 0.05$ between treated and control. Mean ± SD with the same number in a column are not significantly different at $p < 0.05$.

In the present study, the impact of *E. coli* bacterial infection on haemocyte-mediated immune responses was studied in the insect pest *P. americana*. At different hours post-infection, THC counts were taken, analyzed, and compared with control specimens. Results suggested that two hours post-infection, the circulating haemocyte level started to increase, and it reached a significant peak value at five hours post infection and gradually

declined at 24 hours post-infection. This observation was similar to that reported by Mudoi et al. in *P. americana* induced by *Beauveria bassiana*, an entomopathogenic fungus[27]. Among the different haemocytes in the American cockroach, prohaemocytes, plasmatocytes, and granulocytes are the most predominant and play a major role in defense[28-29]. In the present study, prohaemocytes showed a massive decline pattern at 2 hours after infection, and it reached its lowest at five hours post-infection. Interestingly, the prohaemocytes started to recover in the next 24 hours. Among all the haemocytes, granulocytes were found to be the major cell type presenting an elevated cell count in the treated insect group (significant increase at 5 hours post-infection). Plasmatocyte counts showed a non-significant decline due to phagocytic effect on the pathogen and its clearance. The altered morphology of granulocytes was noticed in the treated group. The granulocyte membrane formed extensive filopodial extensions from the plasma membrane to connect with other cells to form an extracellular trap. The cytoplasm of the majority of granulocytes presents vacuolation and membrane blebbing. Awad, 2012 observed the effect of *Bacillus thuringiensis* on the haemocytes of the larval instar of *Agrotis ipsilon* and showed similar results[30]. A study conducted by Ali et al. on *P. americana* brain lysate and haemolymph showed antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*[31]. Identical results were obtained on house crickets after the injection of the pathogenic *E. coli* K1 strain to investigate and characterize the innate immune response by Reginald et al.[32]. Observations on cricket haemocytes infected with *E. coli* by Cho and Cho have shown activation of lysosomes in granulocytes at four hours of infection, reached a peak at 12 hours, and came back to the baseline at 24 hours of infection[29]. The present study suggested unique cellular immune regulation by haemocytes. However, this distinctive mechanism of cell mediated innate response needs further investigation.

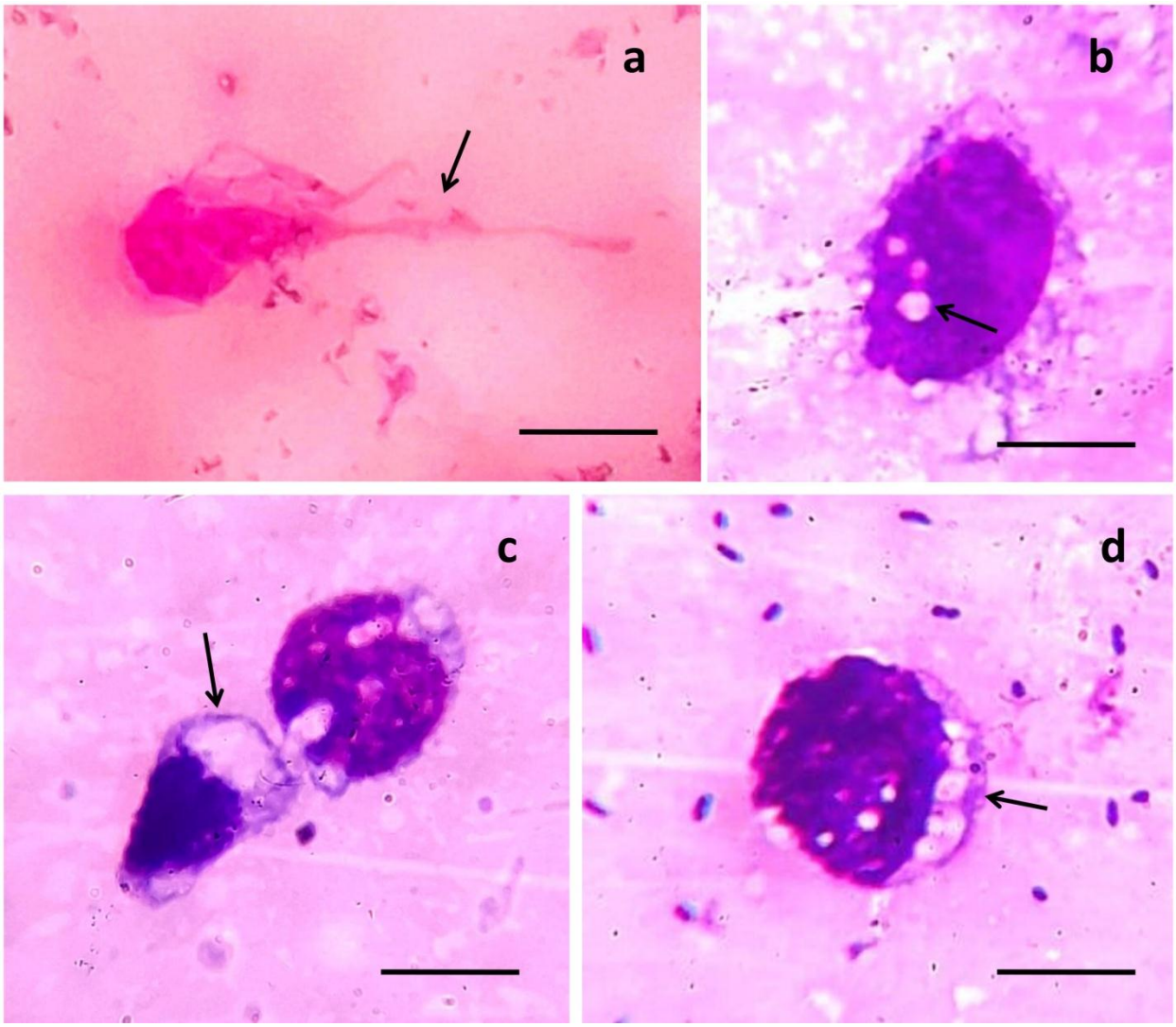

Figure-2: Morphological changes observed in haemocytes of *P. americana* 24 hours after *E. coli* injection. a. Filopodia formation, b. Vacuolation c. Aggregation and vacuolation d. Membrane blebbing in granulocyte.

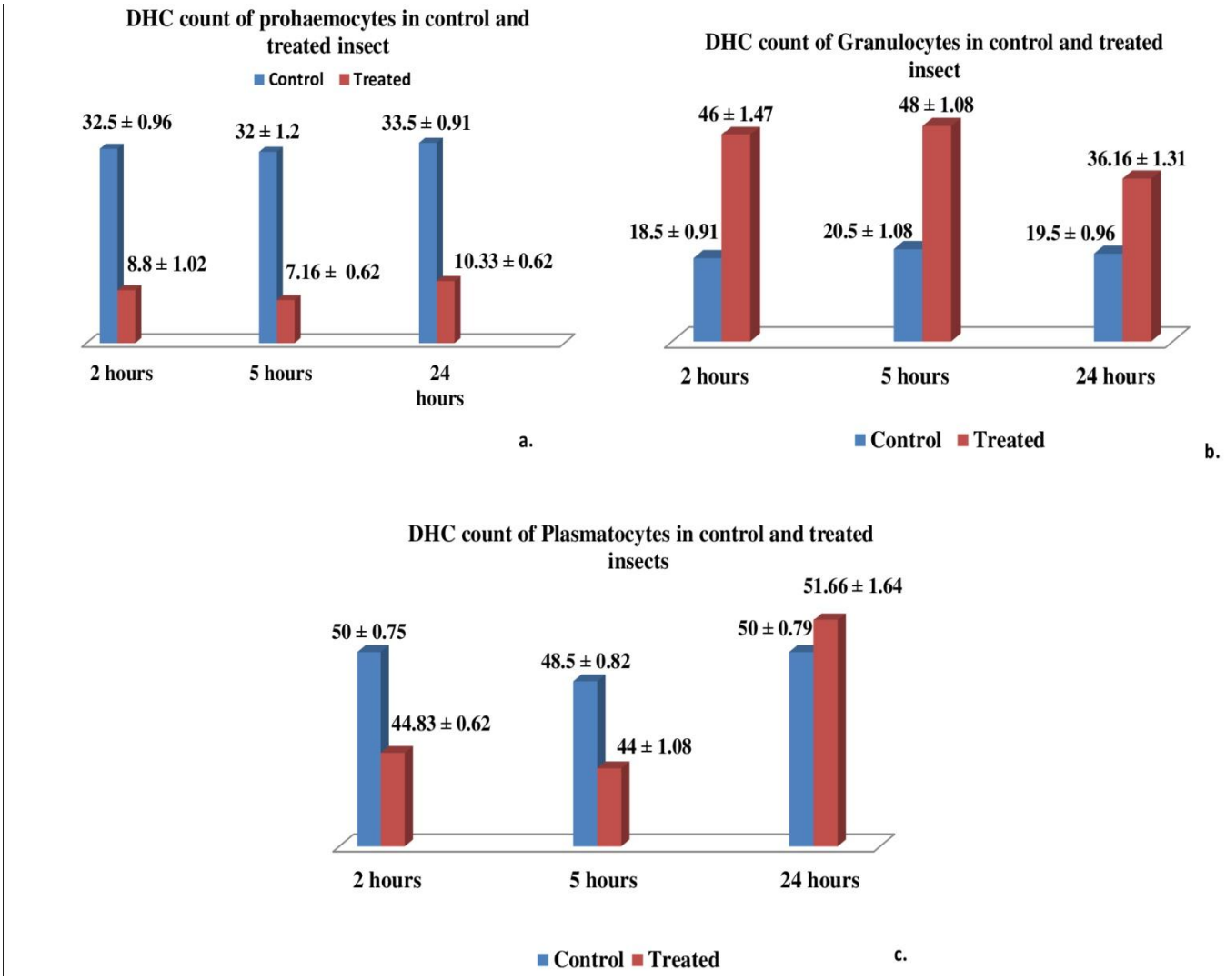

Figure-3: DHC counts of prohaemocyte, granulocyte and plasmatocyte in control and treated insect.

Table-3: Differential hemocyte counts (DHC) in control and treated *P. Americana.*

Hours Post- inoculation	DHC					
	Prohaemocyte		Plasmatocyte		Granulocyte	
	Control	Treated	Control	Treated	Control	Treated
2hours	32.5 ± 0.96[a]	8.8 ± 1.02[b]	50 ± 0.75[a]	44.83± 0.62[a]	18.5± 0.91[a]	46 ± 1.47[a]
	P(T<=t) two-tail: 0.0069		P(T<=t) two-tail: 0.0704		P(T<=t) two-tail: 0.0622	
5 hours	32 ± 1.2[a]	7.16 ± 0.62[b]	48.5 ± 0.82[a]	44 ± 1.08[a]	20.5 ± 1.08[a]	48 ± 1.08[b]
	P(T<=t) two-tail:0.0192		P(T<=t) two-tail: 0.0704		P(T<=t) two-tail: 0.0346	
24 hours	33.5 ± 0.91[a]	10.33± 0.62[b]	50 ± 0.79[a]	51.66 ± 1.64[a]	19.5 ± 0.96[a]	36.16 ± 1.31[a]
	P(T<=t) two-tail:0.0214		P(T<=t) two-tail: 0.4096		P(T<=t) two-tail:0.0957	

Values are expressed as Mean ± SD (n = 3). Mean ± SD with the different letter in a row are statistically significant.

Conclusions

Circulating haemocytes of *P. americana* are important for protection against *E. coli* infection. As numbers of granulocytes increase, it indicates an inflammatory response against bacterial pathogens. A decline in prohaemocyte numbers indicates a mobilization of haemocytes to differentiate into other haemocyte types. Among the three time points of investigation the peak at 5 hours post infection, reflected the optimal response time. In 24 hours post infection possibly a regulatory mechanism prevented excessive haemocyte production.

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