Escherichia coli Challenging Modulate the Humoral Immune Response of Lucilia cuprina (Diptera: Calliphoridae)

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Abstract

Antimicrobial compounds are recently emerging as anti-infectious agents that can be applied topically or systemically to speed up wound healing process. Maggot debridement therapy has become more prevalent in the treatment of chronic wound. This study focuses on studying the activity of commonly used natural compounds, which can be extracted from the whole and fat bodies of Lucilia cuprina. The current report was designed to investigate the effect of injecting bacterial suspension of Escherichia coli during the development of Lucilia cuprina at different time points. Measurements involved the assay of (a) total protease activities, (b) semi-quantitative RT-PCR for selected antimicrobial peptides genes expressions and (c) antioxidant capacity measurement as part of normal responding elements to humoral innate immunity (represented by lipid peroxide (Malondialdehyde) and glutathione reduced levels). Total lipid content in immature stages was also assessed. The results revealed an increase in total proteases activities and antioxidant capacities (low Malondialdehyde and high reduced glutathione levels) during the 3rd instar larvae and pupal phases after bacterial challenge. Detection of an increased mRNA expression levels in the fat body for lysozyme, cecropin, and attacin genes after bacterial-infection indicate high antibacterial activity in early pupae at 1h after infection. In conclusion, our findings may draw attention proposing that naturally extracted compounds from the whole and fat body of Lucilia cuprina, which are rich in proteolytic enzymes, as novel larval-based therapy in parallel to the traditional known ones. Further studies are required to illustrate the exact components of the extracts.

Keywords
Lucilia cuprina; Protease activities; Oxidative stress; Gene expression

Introduction

The calliphorid fly, Lucilia cuprina, is known to cause serious myiasis in animals, whereas its sibling species Lucilia sericata is commonly a carrion breeder and is used in maggots debridement therapy (MDT). Tantawi et al. [1] reported an accidental involvement of L. cuprina in MDT in Alexandria, Egypt, that has proved to be safe and effective. In November 2008, Flies from L. cuprina were successfully used to heal the diabetic foot wounds of two patients at Alexandria Main University Hospital.

It has been reported that the survival of insects in a microbe-thriving environment depends on innate immune responses which is the first line of defense to effectively kill the invading microbes and infectious microorganisms [2]. Basically the humoral immunity in insect involves (a) the induction of proteolytic cascades; (b) the synthesis of antimicrobial peptides; and finally (c) the generation of reactive oxygen intermediates (ROI) [3].

Anti-microbial peptides (AMPs) are among the lines of insect defense that synthesized mainly in the fat body and secreted into the haemolymph to combat a variety of pathogenic microorganisms [2]. Supported by their amino acid composition and antimicrobial activities, AMPs are generally classified into groups [4]. We aimed to investigate the defense mechanism mediated by Attacin, lysozyme, and cecropin during the developmental stages as AMPs within the framework of direct injection of bacteria into the haemolymph of L. cuprina to demonstrate their activity to invading organism.

In most studies a general deficiency of the immune system is related to age, this marked peculiarity is conspicuous in insects. Though the immune response is high in larvae, yet there are specific features such as antimicrobial peptides, cannot be extended to adults. All these aspects of immature stage might cause the high sensitivity of the larvae and pupae to simple aseptic injury with bacteria [5].

In this report we aimed to investigate the importance of the fat body as being an effective component, by determining the total lipid content at selected developmental stages. The results obtained herein reflect the intention to demonstrate which immature stage that has a relatively high level of protease peptides, antioxidant compounds, and antibacterial proteins. Our objective was to propose biologically active molecules from maggots and early pupae to utilizing their extracts to be applicable therapeutically in the future, aiming for medicinal therapy could be then replaced by these active molecules, either in their native or synthetic form, assuming that they may help to understand the mechanisms underlying the healing properties of maggot therapy and its application.

Materials and Methods

Laboratory rearing and maintenance of the colony

The colony of the blowfly L. cuprina was reared and maintained in the insectary laboratory of Faculty of Science at Alexandria University.

Larvae of L. cuprina were fed on beef liver to adulthood. Upon eclosion artificial diet of 50:50 mixtures of table sugar and powdered milk was introduced to the adults. This stock of blowflies was best maintained with minimal light at 30°C with 80% relative humidity.

Bacteria

Escherichia coli (ATCC) strain was selected to be used for infection. The bacteria were cultured in 10 ml Luria-Bertani (LB) medium at 37°C until the absorbance at optical density OD600 reached 0.5 [6].

Injection procedure

Larvae were immobilized by chilling; selected samples in their mid-stage (~13-16 h) were then injected with a suspension of 1 µl E. coli.
coli suspended in LB media (0.5 OD_{600}) into the terminal abdominal segments. Negative control was injected with LB alone as an empty vehicle. Each group of larvae was then placed in a separate beaker at 30°C then collected at time intervals of 1, 8, and 16 h after the infection. Pupae were also isolated at early-, mid- and late-stages i.e. approximately of 1-5, 40-45, and 80-85 hours respectively. Replicates of pupae were micro-injected with bacterial concentrations of (0.5 OD_{600}), and placed in separate beaker as indicated above.

Isolation of fat bodies

The fat bodies were isolated from control, induced larvae and pupae at 1, 8, and 16 h after induction. The fat body samples were kept at -80°C for RNA extraction.

Homogenization of whole bodies' samples

Insect samples were washed with distilled water then with 70% (v/v) ethanol for sterilization and stored frozen at -80°C until future use. Samples were subsetablemente homogenized with test-specific buffer according to the type of assay performed.

Measurement of total lipid content

The lipid fraction in larvae and pupae were extracted and total lipid contents were determined using the method described by Zöllner and Kirsch [7].

Protease activity assay - albumin as substrate

The assay was performed according to method described before [8] with minor modifications. Briefly, tissues were homogenized in Tris–HCl buffer (pH 7.5), then 2 µl of stock albumin solution (4 g/dl) and 50 µl of sample homogenate (0.1 g/100 µl) were mixed, left to stand at room temperature for 10 min, then 1 ml of color reagent is added followed by well mixing. The absorbance was measured after 5 min that corresponds to the un-reacted albumin. Into another clean test tube, 2 µl of stock albumin and 1 ml of color reagent are well mixed, left to stand at room temperature for 5 min, then 50 µl of sample is added. The absorbance measured after 5 min at 630 nm corresponds to total albumin. Calculations were made according to the following equations:

Reacted albumin = total albumin – unreacted albumin

Reacted albumin is equivalent to the total proteases activity of the sample.

Reduced Glutathione (GSH) level

The concentration of GSH was determined as described previously [9]. Briefly, 0.5 ml of the tissue homogenate was mixed with 0.5 ml of 500 mmol/L Trichloroacetic acid (TCA) solution and allowed to stand for 5 min at RT then centrifuged at 3000 rpm for 15 min. Finally 0.5 ml of the supernatant was mixed with 1.0 mmol/L DTNB (5,5 Dithiobis-2-Nitrobenzoic Acid) and 100 mmol/l sodium citrate. GSH concentration was calculated from the measured absorbance at 405nm and presented as mg/g. tissue = A sample × 66.66/g. tissue used.

Determination of Malondialdehyde (MDA) concentration

The process of lipid peroxidation results in the formation of MDA. This is a later product in the sequence of lipid peroxidation reactions. Lipid peroxidation (LPO) assays were carried out according to Kei [10] method. MDA concentration is calculated as nmol/g. tissue = (A sample / A standard) X (10 / g. tissue used).

RNA extraction

Total RNA from fat and whole bodies of blowflies L. cuprina were extracted according to previously described [11] procedure using GStractTM RNA Isolation Kit II Guanidinium Thiocyanate. RNA concentration was determined by UV spectrophotometry at 260 nm, and the purity of the RNA preparation was estimated according to the ratio of absorbance readings at 260 nm and 280 nm [A 260/A 280] with optimum ratios between 1.8 and 2.0.

Semi quantitative reverse transcription-PCR (SemiqRT-PCR)

Semi-qRT-PCR was performed using two-step RT-PCR method. cDNA synthesis is carried out using RevertAid First Strand cDNA Synthesis Kit following the manufacturer guidelines. In brief, 3 µg RNA was added into reverse transcriptase and RT buffer and incubated at 45°C for one hour for Reverse Transcription, then at 83°C for enzyme inactivation as described in the kit pamphlet.

Subsequently 300 ng from the synthesized cDNA was then used for each PCR cycles for the amplification of L. cuprina target genes (lysozyme, cecropin, and attacin). The specific primer set is added to each tube separately. Primers were designed using primer 3 software as shown in Table 1.

The PCR conditions were adjusted for each set of primers according to the optimum annealing temperature. Each cycle consist of denaturation, annealing and extension steps and total number of 35 cycles were used.

Agarose gel electrophoresis of the amplified RT-PCR products

According to Robyt and White [12] method, RT-PCR products were separated on agarose gel. Mixture of total volume of 12 µl was prepared by mixing 10 µl of the RT-PCR product and 2 µl of sample loading dye and electrophoresed on 1.5% agarose gel (1.5g/100 ml 0.5x TBE) containing 10 µg/ml Ethidium bromide (EtBr). Samples were prepared in loading dye [0.089 M Tris base, 0.089M boric acid, 0.002M EDTA, disodium sale, dihydrate, pH 8.3]. The resulted bands were visualized using the ChemiDoc-It2 Imaging System.

Statistical analysis

All measurements were made either in triplicate or duplicate. For calculations of significance, the results were analyzed using one-way ANOVA (Analysis of Variance) SPSS program to determine differences between groups (P ≤ 0.05).

Results and Discussion

This study was designed to investigate the effectiveness of L. cuprina antibacterial activity, as part of its humoral immune response.
upon stimulation by injection of bacteria. Direct bacterial injection into _L. cuprina_ haemolymph was performed to test the insect defense against invading organism. The optimal induction conditions for the antimicrobial compounds production in _L. cuprina_ after infection with _E. coli_ (Gram-negative bacteria) were optimized in the 3rd instar larvae, early pupae, mid pupae, and late pupal phases, at selected time interval of 1, 8, and 16 hours. This species of the Calliphoridae family may furnish a new species to be applicable as maggot therapy (MT). The antibacterial components (protease peptides, antioxidant compounds, and antibacterial proteins peptide genes) from whole body extract of selected phases revealed promising results assessed with each technique used here, permitting this study to add _L. cuprina_ as an alternative model to be adapted in maggot therapy, in competition to _L. sericata_.

Arrese and Soulages [13] have valued how insects utilize lipid for the production of immune-reactive components that could be used as an energy source and/or for membrane biogenesis in the sites of infection or in haemocytes. Accordingly this explains herein the significant decrease of total lipid content of selected immature stages of _L. cuprina_ after being exposed to stress, inoculated with gram-negative bacteria _E. coli_. In addition, the data displayed, that the total lipid content decreases during the pupal phase compared to the 3rd larval instar and the 3rd instar showed the higher activity (Figure 1), which may be attributed to the fact that fat body phosphorylase activity increases during larval development, and prior to pupation. The decrease of lipid may be linked to the need of energy for chitin synthesis.

Maggots secrete a mixture of proteolytic enzymes that may act as debridement agents, degrade a variety of extracellular matrix components (ECM), promote human dermal fibroblast migration and accelerate extracellular matrix remodeling [14]. The experimented insects infected with _E. coli_ manifested a conspicuous increase of the total protease activity reducing with time (1h to 16h) through studied phases. These results support the hypothesis that antibacterial activity of infected insects increased in a few hours then sharply decreased, which may attribute to the immune response (Figure 2). Similarly, Feder et al. [15] documented that the up-regulated expression in metalloproteinases that are produced in the fat body of a blood sucking
insect *Rhodnius prolixus* after infection. In addition, the expression of genes encoding protease enzymes has been detected as a response to septic wounding of sterile second instar larvae of *L. Sericata* [16].

Lipid peroxidation is a chain reaction between polyunsaturated fatty acids and Reactive Oxygen Species (ROS), and it produces lipid peroxides and hydrocarbon polymers that are both highly cytotoxic [17], leading to oxidative damage and alterations in radical scavenging enzymes in insect tissues [18]. Broderick et al. [19] detected higher levels of ROS from *Drosophila* haemolymph of *Serratia marcescens* exposed larvae compared to the control group, which is in agreement with our study results that reported a significant increase in Malonyldialdehyde concentration at 1 and 8 hours post-infection with *E. coli* in *L. cuprina* larvae as compared to non-infected controls. This may indicate that *E. coli* induced high lipid peroxidation that leads to cytotoxicity in infected larvae and pupae (Figure 3).

The non-ribosomal antioxidant tri-peptide, GSH, is a key component of the cellular defense against injury and lipid peroxidation damage and a co-factor for the activity of Glutathione S-Transferase (GST). These have a crucial role in the termination of free-radical cascades and the lipid peroxidation chain reaction [20], which explains why GSH increase with lipid peroxidation in this study. The current results were in agreement with Parkes et al. [21] who showed that GST and GSH play a vital role in prevention and repair of oxidative damage by detoxifying lipid peroxidation products in *Drosophila melanogaster*. The coordination of GSH biosynthesis, GST expression and glutathione S-conjugate functions have a key role in the antioxidant defense of higher vertebrates. Our study is in agreement with all previous reports which demonstrate that enhanced activities of GSH can lead to the elimination of ROS (Figure 4).

Around 30 genes have been cloned in *Drosophila* and 47 genes were identified in *L. sericata* encoding anti-microbial peptide (AMPs) displaying activities against microorganism, such as *Lucilia* defensin, diptericin and proline-rich AMPs [16,22-23]. The antibacterial peptide genes expression of *L. cuprina* for which investigated in this study was expressed in control and under bacterial-challenging of different developmental phases. Our results showed that during larvae and pupal phases of *L. cuprina* the expression levels of three antibacterial genes (lysozyme, cecropin, and attacin) displayed different transcript patterns (Figure 5). Each AMP has a potential activity against a range of microorganisms. Lysozymes possess high activity against gram-positive bacteria, by hydrolyzing the 1, 4-β-linkage between N-acetylglucosamine and N-acetylmuramic acid of the cell wall peptidoglycans [24]. A perception was observed by Boma et al. [25] they noted that lysozyme is bactericidal to only gram-positive bacteria like *Bacillus megatherium*, and *Micrococcus luteus*. Vieira et al. [24] have assigned in their studies that gram-negative bacterium *E. coli* had still an immunological role of lysozyme gene expression, showing a synergistic effect between lysozymes and other
Figure 5: RT-PCR for detecting lysozyme gene transcripts from the whole and fat bodies extracts of L. cuprina at 3rd instar larvae after E. coli-infection (E.coli), compared to Control at 1h, 8h, and 16h. (A) Expression pattern of lysozyme gene and (B) the intensity of bands were measured and plotted on graph using Quantity One software (ImageJ).

Figure 6-a: Reverse transcriptase-PCR analyses of the lysozyme gene transcripts from the fat body extract of L. cuprina 3rd instar larvae, early-, mid-, and late pupae, respectively after E. coli-infection (E.coli), compared to Control (C) at 1h, 8h, and 16h. Expression pattern of lysozyme gene (A) and the intensity of bands were measured and plotted on the graph using Quantity One software (ImageJ) (B).

Figure 6-b: The time-course expression analysis of L. cuprina lysozyme gene by RT-PCR for 3rd instar larvae, early-, mid-, and late pupae, respectively after E. coli-infection (E.coli), compared to Control (C) at 1, 8, and 16h, respectively. Statistical analyses of 2 parallel RT-PCR result of L. cuprina genes expression were performed. Error bars represent the S.D. of 2 independent PCR amplifications and quantifications (n=2). The relative expression levels were expressed as the ratio of each gene to GAPDH calculated from the image using Quantity One (ImageJ software). The asterisks ***, **, and * indicate significant differences in L. cuprina genes expression levels post- bacterial challenge compared to control at levels of 0.001, 0.01, and 0.05, respectively.
AMPs enhancing the immune responses against both gram-positive and gram-negative bacteria. It was noted herein that the expression of lysozyme gene after induction with gram-negative bacteria (E. coli) was up-regulated when analyzed by SemiquRT-PCR (Figures 6-a and 6-b).

Cecropins, a family of small antibacterial peptides, exhibit a broad spectrum of activity against gram-positive and gram-negative bacteria and have been isolated from many insects. Cecropins can also kill some pathogenic microorganisms at nano-molar concentrations with little effect on normal eukaryotic cells, they kill the microorganisms within minutes through lytic/ionophoric mechanism [25]. The protective function of Cecropin in Calliphora vicina varies according to the developmental stage; organisms that had just pupated and were not fully tanned exhibited the greatest response to the administered insult by E. coli. The exhibition of this high immune response is due, in part to the high concentrations of Cecropin manufactured at this instar of the fly’s life cycle [26]. The results obtained herein are for the early pupal phase, showing that the cecropin gene was significantly expressed in the bacterial-challenged samples at different developmental stages when compared to control (Figures 7-a and 7-b).

Attacins are glycine-rich immune proteins [4], which was isolated from various insect species, such as Diptera and Lepidoptera species [27]. In this study, the investigation of the transcription profile of attacin gene in different developmental stages of L. cuprina showed no significant difference between infected and non-challenged samples except in the early pupal stage which exhibited the greatest response to E. coli infection at 1h. These results suggest that attacin may not be able to kill E. coli bacteria, which is consentient with a discrepancy that was explained by the finding that attacins only act on growing bacteria [25]. Herein, it may be suggested that the exhibition of high expression of attacin in early pupae is correlated particularly to its high significant, due to that attacin facilitates the action of cecropin and lysozyme, thereby enabling these three immune proteins to work in consonance (Figures 8-a and 8-b).

It is well known that the major site of synthesis of immune-induced antimicrobial peptides in insects is the fat body that might play certain roles in the physiological processes and immune regulations, besides...
the gut cells and salivary gland cells [22,28] showed that Drosophila responds to a septic injury by the rapid synthesis of antimicrobial peptides, these molecules are predominantly produced by the fat body, a functional equivalent of mammalian liver, same results were ascertained in the current study, when comparing the significance of lysozyme in fat body to whole body extract, it was noted that lysozyme was highly significant in fat body rather than the whole body extract in L. cuprina, which coincides to previous research [6], as they showed that lysozyme content in the fat body and haemolymph was lower than that in the midgut of Musca domestica [203]. A possible explanation for this discrepancy could be also attributed herein to that direct injection of bacteria into the haemolymph (septic injury) elicits a systemic immune response in the fat body, while oral ingestion of bacteria elicits the immune response in the gut epithelium [29].

The data obtained through this study is still in its infant phase, aiming to predict certain candidates of antibacterial peptides and proteases, which could participate in the positive effects of larval therapy. Further, it will be possible to prepare such peptides and proteins using recombinant techniques and to test their activities using in vitro assays separately and not as a part of a mixture. This approach will allow us to better understand the mechanism of how larvae are able to eliminate various pathogens and generally enhance the healing process. Active recombinant peptides and proteins could be used in the future as new bio-therapeutics agents.

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Conflicts of Interest
The authors declare that they have no competing or conflicts of interests.

References


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