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Enhancing the Humoral and Melanization Responses of *Aedes aegypti* Mosquito: A Step Towards the Utilization of Immune System Against Dengue Fever

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Abstract: Great efforts are currently being done to utilize the immune system of mosquito vectors in the battle against the different mosquito-borne parasitic and viral diseases. Based on this control strategy, the current study has been conducted to induce and enhance the most effective immune responses, the humoral and melanization responses, in the dengue fever vector, *Aedes aegypti* against live bacteria and non-biological agents at 24 h post-treatments. The humoral activity against *Bacillus subtilis* and *Escherichia coli* was investigated after thoracic injection of the same bacteria or the lipopolysaccharide (LPS) into mosquitoes using the inhibition zone assay. Melanization response was tested against the thoracic inoculated Sephadex® beads, positively charged CM A-25, negatively charged CM G-25 and neutral CM C-25 and inert glass beads. These immune responses were then enhanced via the oral administration of 0.3% thymoquenone (Thq), the main active ingredient of the black seeds, *Nigella sativa*, to mosquitoes. Data demonstrated that, on one hand mosquitoes exhibited strong humoral activity against the injected bacteria as well as against the well-known immune sensitive bacteria, *Micrococcus luteus.* Moreover, melanization response was strong against differently charged Sephadex® beads, but not against the inert glass beads. On the other hand, significant increases in the humoral anti-bacterial lyses activity and anti-beads melanization response (up to 6 folds in some cases) was clearly shown when mosquitoes were maintained on 0.3% Thq-glucose mixture (in 10% glucose). Therefore, these data may indicate that both humoral antimicrobial activity and melanization response could be enhanced to be more effective against disease-organisms transmitted by mosquitoes. Thus, this study suggests a possible immuno-control strategy in the battle against mosquito-born diseases which, in fact, will be tested against dengue viruses in the future studies.

Key words: *Aedes aegypti*, dengue, immuno-control, melanization, humoral activity

INTRODUCTION

Insect-borne diseases are major causes of death and morbidity throughout the world. Mosquitoes of the genus *Aedes* (Order Diptera, Suborder Nematocera, Family Culicidae, Subfamily Culicinae) are considered disease vectors as they are responsible for the transmission of a number of viral and parasitic human pathogens worldwide. This is because this genus has a cosmotropical distribution between 30°N and 20°S (Christophers, 1960; Knight and Stone, 1977) and exhibits a distinct preference for human habitats, including artificial oviposition sites, e.g., tires, flower vases and water storage containers (Tabachnick, 1991). *Aedes aegypti* is considered the primary, worldwide arthropod vectors for yellow fever and dengue fever flaviviruses worldwide (Gubler, 2002). An estimation of 2,500 million of people distributed within 100 countries, live in tropical and subtropical areas, are at risk of epidemic dengue virus transmission (World Health Organization, 2006).

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Mosquito-borne diseases are considered human health problems due to two main factors; a) although attenuated candidate vaccine viruses have been developed recently (Robertson *et al*., 2002; Shepard *et al*., 2004; Meeting Report, 2005), it is still no effective dengue vaccine is available and b) emergence of resistance in mosquito vectors to most of chemical-insecticides. Hence, three recent novel strategies are currently being suggested as alternatives for blocking dengue transmission; the first strategy, is utilizing the suitable bio-control agent, for example, the *Mesocyclops* copepods against mosquito larvae (Kay *et al*., (2002). The second strategy, is advocating the genetic engineering to develop novel mosquito-control methods to improve the existing biological control strategy. The third strategy is developing resistance in the vector to block the virus transmission by genetically selecting dengue-refractory strains of *Ae. aegypti* (Wallis *et al*., 1985; Miller and Mitchell, 1991; Olson *et al*., 2002; Travanty *et al*., 2004). This refractoriness is commonly referred to as a midgut infection barrier (preventing replication of the virus in the gut epithelium) and midgut escape barrier (preventing releasing of verions from midgut epithelium to salivary gland), respectively (Black *et al*., 2002).

Ongoing great efforts are currently being done to utilize the immune system of mosquito vectors in the battle against different mosquito-borne diseases (Kohoza *et al*., 2000). This strategy is being seriously tested regardless the reproductive cost, the price of immune induction, that must be paid by the vector (Yan *et al*., 1997; Irvin *et al*., 2004). However, a less-costly immune responses have been induced in the African malaria vector, *Anopheles gambiae*, via the oral administration of black seed oil, a natural botanical extract from *Nigella sativa*, to mosquitoes (Ahmed, 2006). From the point of view of dengue virus transmission, Gorrochotegui-Escalante *et al*. (2005) divided dengue-infected mosquito into 3 categories; a) susceptible, those with an infected head, b) those had an infection barrier, without a midgut infection and c) those had an escape barrier, with an infected gut but no head infection. From the point of view of mosquito immunity, there is very limited knowledge of mosquito immune responses to viral infection. However, much is known about the involvement of mosquito innate immunity in bacterial and fungal infections (Scholte *et al*., 2007; Christophides *et al*., 2002), but few studies have analyzed the role of these immune responses in arthropod-borne viral infection (Sandersa *et al*., 2005; Brown and Hancock, 2006; Büyükgüzel *et al*., 2007).

Interestingly, mosquito cells have been found evolving mechanisms to specifically control RNA-virus replication in its body cells. This occurs as host cells detect double stranded RNAs (dsRNAs) that form during RNA virus replication. Thus, recent molecular control strategies rely upon blocking the viral transmission by expressing specific anti-dengue virus effector molecules throughout the whole mosquito or preferentially in the salivary glands or midgut of the transgenic mosquito (e.g., Olson *et al*., 2002) for blocking replication and transmission of the viruses (Sanchez-Vargas *et al*., 2004). Thus, the challenge is to utilize these strategies to silence virus replication at critical sites in the vector such as midgut and salivary glands. Thus, the idea of the current study is based on that immunological manipulation of *Ae. aegypti* mosquitoes may profoundly and permanently reduce their vectorial capacity. This hypothesis is based on that the successful transmission of a pathogen to its vertebrate host reflects the culmination of a complex series of events within the mosquito vector as it must overcome an arsenal of internal active defence mechanisms dedicated to the recognition and subsequent destruction of non-self (foreign) entities (Barillas-Mury *et al*., 2000; Lowenberger, 2001). In order to utilize these immune responses to efficiently block viral transmission, we should thoroughly understand the processes of defense responses in the mosquito vector.

Thus, the hypothesis underlying the current study is to stimulate and enhance the non-self entities of the dengue vector against biological and non-biological agents, which assumed to be exhibited against dengue virus in nature. This hypothesis is based on three preliminary steps; a) studying the humoral antimicrobial activity of the vector against injected both LPS and the challenged live bacteria, *B. subtilis* and *E. coli*, b) studying the humoral melanization response against inoculated Sephadex® beads of different charges and c) Investigating the enhancive effect of thymoquenone (Thq), the main

active ingredient of the black seed oil (*Nigella sativa*), on these immune responses. This hopefully may help improving the immuno-control strategy in the battle against mosquito-borne diseases in general.

MATERIALS AND METHODS

Establishment of the Experimental Mosquitoes

The proven dengue vector, *Ae. aegypti*, in KSA have been selected for this study as it is a native mosquito in Jeddah regions. This dengue vector was collected from Jeddah and reared in the insectary of zoology Department, Faculty of Sciences, University of King Saud, under standard conditions as outlined in Ahmed *et al*. (1999) to ensure that mosquitoes of the same size are produced (checked by measuring wing lengths). Adults emerging within a 24 h period were maintained in the rearing cages with permanent access to a 10% glucose solution (W/V). Mosquitoes of the same age post-emergence were used for the experimental purposes of this study. To maintain a stock of mosquito colony, they were routinely fed upon the blood of an anaesthetized mouse in order to lay eggs for new generations.

Oral Administration of Thymoquenone

The main active ingredients of the black seed oil, thymoquenone (Thq) was used in this study as a natural immune-enhancing agent. Based on a previous study (Ahmed, 2004), 0.3% Thq (v/v) [mixed with 10% glucose solution (w/v)] was fed to mosquitoes. Experimental mosquitoes were allowed to feed on Thq-glucose mixture or 10% glucose solution (w/v) immediately after adult emergence and until the end of experiments. The enhancive effect of Thq on humoral and melanization responses was studied in blood fed mosquitoes (maintained on 0.3% Thq-glucose mixture or 10% glucose).

Bacterial Injection, Haemolymph Collection and Inhibition Zone Assay

The mosquito toxic and non-toxic bacteria, *Bacillus subtilis* (Geetha and Manonmani, 2008) and *Escherichia coli* respectively that used in this study were kindly provided by Department of Food Sciences and Nutrition, Faculty of Food Sciences and Agriculture University of King Saud and prepared for mosquito inoculation as detailed in Nimmo *et al*. (1997). For inoculation, mosquitoes were immobilized on ice and sham injected with 0.25 µL of *Aedes* physiological saline (APS) (13 mM NaCl, 0.5 mM KCl, 0.1 mM $CaCl_2$) or lipopolysaccharide (LPS) (10 ng/0.25 µL APS/mosquito) into the thoracic haemocoel using a calibrated hand-made heat-pulled microcapillary needle as detailed in Ahmed *et al*. (2002), or inoculated with bacteria by pricking (injection) with a fine capillary needle (dipped in a concentrated suspension of bacteria) according to Dimopoulos *et al*. (1997). Any mosquito that was severely bled after inoculation was discarded from the study. After pricking, mosquitoes were allowed to recover and maintained in cages (16×16×16 cm) in standard insectary conditions. No difference in the mortality of the injected or sham-injected mosquitoes was observed (data not shown). Haemolymph was collected from the thoraces of experimental mosquitoes 24 h post-treatment and subjected to an inhibition zone assay as detailed in Ahmed *et al*. (2002), to measure the humoral activity against each injected bacteria. For comparison, the humoral activity against the immune-sensitive bacteria, *Micrococcus luteus* (NCTC 2665, Sigma, UK) was tested as a result of injection with LPS, *B. subtilis* or *E. coli* bacteria. Five replicates in each case (from five different mosquitoes) (n = 5) were carried out to perform statistical analysis.

Inoculation of Sephadex® Beads

Positively charged CM A-25, negatively charged CM C-25, neutral CM G-25 Sephadex® beads (Sigma-Aldrich, UK) and inert glass beads were re-hydrated in APS prior to thoracic inoculation into mosquitoes according to Koella and Sørensen (2002). Inoculated mosquitoes were then maintained in small cages (16×16×16 cm), kept in their rearing insectary and allowed access to 0.3% Thq-glucose

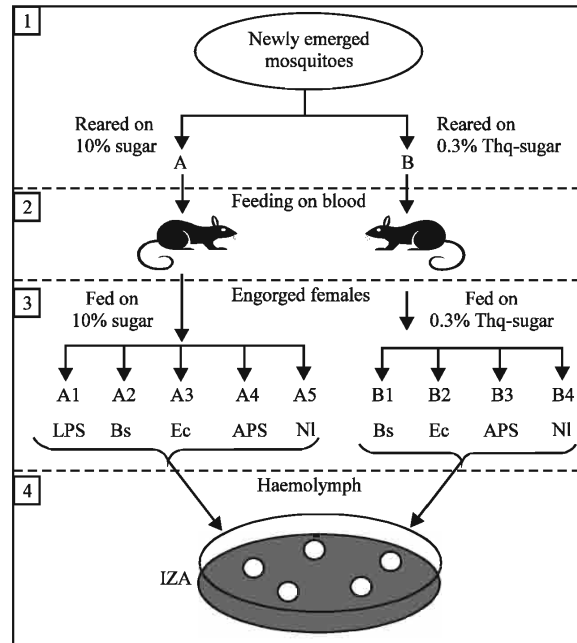

Fig. 1: An illustration showing the experimental design for inducing and studying the effect of Thq on the humoral antimicrobial activity, in *Ae. aegypti*, (1) Newly emerged mosquitoes were divided into groups A and B (˜ 300 mosquitoes each) and was immediately allowed access to 10% glucose solution and 0.3% Thq-glucose mixture respectively until the end of experiment, (2) Six days old mosquitoes from both groups were allowed access to blood meals from anaesthetized CD mouse for 20 min, (3) After blood feeding, fully engorged females from groups A or B were randomly selected and divided into the subgroups A1 to A5 or B1 to B4, respectively (30 mosquitoes each) in a small cage (16×16×16 cm). The subgroups A1, A2, A3, A4 and A5 were immediately injected with LPS, *B. subtilis* (Bs), *E. coli* (Ec), APS or left non-injected (NI), respectively. The subgroups B1, B2, B3 and B4 were immediately inoculated with Bs, Ec, APS or left non-inoculated (NI), respectively. Treated groups were then allowed access to their relevant sugar solutions again until 24 h later, (4) At 24 h post-treatments, haemolymph from subgroups A1 to A5 was collected and used freshly for inhibition zone assays (IZA) in agar seeded with Bs, Ec or *M. luteus* bacteria for comparison. While Haemolymph from subgroups B1 to B4 was tested against Bs or Ec only. Five replicates (n = 5) from each groups were done to perform statistical analysis.

mixture while control group were allowed access to 10% glucose solution. At 24 h post-inoculation, only active mosquitoes (that were capable of moving and flying) were used for dissecting their thoraces for monitoring beads melanization. According to Koella and Sørensen (2002), beads melanization was scored in three degrees; no visible melanization (white bead), patchy (dotted or half melanized bead) and complete melanization (dark black beads) (Fig. 8). Percentages of mosquitoes that showed each melanization degree were then calculated (in 10 mosquitoes from each group) (n = 10).

Experimental Protocols

To test whither or not Thq has an enhancive effect on both humoral and melanization responses in the dengue fever vector, *Ae. aegypti*, the following experimental designs have been undertaken:

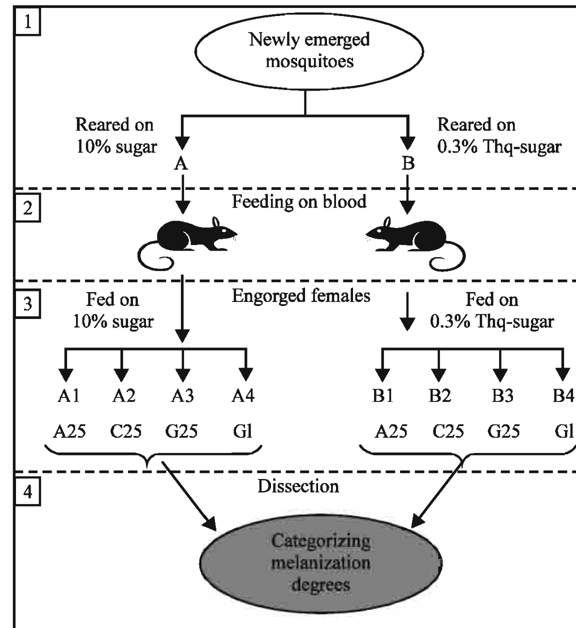

Fig. 2: An illustration showing the experimental design for inducing and studying the effect of Thq on melanization response against inoculated Sephadex® beads in *Ae. aegypti*, (1) Newly emerged mosquitoes were divided into groups A and B (˜ 300 mosquitoes each) and immediately allowed access to 10% glucose solution and 0.3% Thq-glucose mixture respectively until the end of experiment, (2) Six days old mosquitoes from both groups were allowed access to blood meals from anaesthetized CD mouse for 20 min, (3) After blood feeding, fully engorged females from groups A or B were randomly selected and divided into the subgroups A1 to A4 or B1 to B4 respectively (30 mosquitoes each in a small cage (16×16×16 cm). The subgroups A1, A2, A3, A4 or B1, B2, B3 and B4 were immediately inoculated with CM A-25, CM C-25, CM G-25 Sephadex® beads and the inert glass beads (Gl) as shown in the figure. Treated groups were then allowed access to their sugar solutions again until 24 h later, (4) At 24 h post-treatment, mosquitoes were dissected 24 h post-inoculation for monitoring beads melanization degrees. Ten replicates (n = 10) from each groups were done to perform statistical analysis

Humoral Antibacterial Activity and Effect of Thymoquenone

As shown in Fig. 1, newly emerged mosquitoes were divided into group A and B which maintained on 10% glucose solution and 0.3% Thq-glucose mixture respectively until the end of experiment. Six days old mosquitoes were starved for 12 h prior to blood feeding on an anaesthetized CD mouse for 20 min. Immediately after feeding, fully engorged mosquitoes of group A or B were randomly selected and subdivided into 5 subgroups (A1 to A5) or 4 subgroups (B1 to B4) (30 mosquitoes each), respectively. Mosquitoes were then intra-thoracicaly injected with filter sterilized APS (sham injected), LPS (10 ng/mosquito) (Sigma, UK), *B. subtilis* (Bs) or *E. coli* (Ec) as shown in Fig. 1. Control mosquitoes were left non-injection (naïve) (NI) as negative control. Injected mosquitoes were then maintained in small cages and allowed access to 10% glucose or 0.3% Thq-glucose mixture again until the end of experiment (Fig. 1). At 24 h post-injections, haemolymph was collected from 5 active mosquitoes (have the ability to fly) and used freshly for inhibition zone assay as described earlier.

Melanization Response and Effect of Thymoquenone

Newly emerged mosquitoes were divided into group A and B which maintained on 10% glucose solution and 0.3% Thq-glucose mixture respectively until the end of experiment (Fig. 2). Six days-old mosquitoes were starved for 12 h prior to blood feeding on an anaesthetized CD mouse for 20 min. Immediately after feeding, fully engorged mosquitoes of group A or B were randomly selected and subdivided into 4 subgroups (A1 to A4) or (B1 to B4) (30 mosquitoes each) respectively. Groups A1 to A4 and B1 to B4 were inoculated with positively charged CM A-25, negatively charged CM C-25, neutral CM G-25 Sephadex® beads or the inert glass beads (Gl) (Sigma-Aldrich, UK) as shown in Fig. 2. At 24 h post-inoculation, mosquitoes were dissected for monitoring beads melanization as described earlier.

Only one bead (suspended in 0.25 μL APS) was inoculated into the thoracic haemocoel (one bead/mosquito) in 0.25 μL APS/mosquito. Beads inoculated mosquitoes were then maintained in small cages as detailed before and allowed access to the 0.3% Thq-glucose mixture again (Fig. 2). Ten active mosquitoes (n = 10) were dissected at 24 h post-treatment for monitoring beads melanization as described earlier. The three melanization degrees were scored according to Koella and Sørensen (2002) in 10 mosquitoes (n = 10) from each mosquito group.

Statistical Analysis

All statistical analyses were undertaken using MINITAB software (MINITAB, Stat College, PA, v. 13.1, 2001). Data of each experiment were first tested for normality and variances homogeneity prior to any further analysis with the suitable test. Because data were non-parametric, Kruskal-Wallis test was used to determine the overall effects of treatments prior to the individual comparisons using the non-parametric test, Mann-Whitney U.

RESULTS

In this study, six days old mosquitoes (maintained on 10% glucose solution or 0.3% Thq-glucose mixture) were used for inducing humoral activity against injected LPS, *B. subtilis* and *E. coli*, or melanization response against charged or neutral Sephadex® beads and inert glass beads. Experimental mosquitoes were verified to have similar body sizes (based on wing lengths measurements) within each experiment (Table 1) in each experiment (Fig. 1, 2).

The Humoral Anti-Bacterial Activity

Humoral activity in the haemolymph of *Ae. aegypti* was first tested against *M. luteus* at 24 h post-injection with LPS and challenge with alive *B subtilis* and *E. coli*. This activity was equivalent to the activity of 341.0±19.0, 132.0±14.0 and 229.0±9.14 ng μL^{-1} haemolymph, respectively (Fig. 3, 4). Mann-Whitney U-test showed that these lysozyme equivalent activities were significantly higher than that of the sham injected or non-injected mosquitoes (v 67.20±14.9 or 49.85±9.14 ng μL^{-1} haemolymph, respectively) ($p<0.5$, n = 5) (Fig. 4). Furthermore, Haemolymph from mosquitoes injected with LPS, *B subtilis* and *E. coli* showed anti *B. subtilis* humoral activity which was equivalent to 191.7±19.0, 161.84±9.14 and 229.0±9.14 ng μL^{-1} haemolymph, respectively (Fig. 5). These activities were significantly higher than that of the sham injected or non-injected mosquitoes (v 79.71±7.1 or 57.31±7.47 ng μL^{-1} haemolymph, respectively) ($p<0.5$, n = 5) (Fig. 5). Finally, Haemolymph from mosquitoes injected with LPS, *B subtilis* and *E. coli* showed anti *E. coli* humoral activity which was equivalent to 453.0±69.4, 161.84±9.14 and 199.16±9.14 ng μL^{-1} haemolymph, respectively (Fig. 6). These activities were significantly higher than that of the sham injected or non-injected mosquitoes (v 94.64±7.47 or 64.8±11.8 ng μL^{-1} haemolymph respectively) ($p<0.5$, n = 5) (Fig. 6).

Table 1: Wing length measurements of mosquitoes used in the studies of humoral activity and melanization response (Fig. 1, 2). Wing lengths of mosquitoes were first measured to ensure similar body sizes before any further treatments. Data were first tested for normality using Anderson-Darling test prior to Mann-Whitney U-test, which showed no significant differences in wing lengths between mosquito groups of each experiment

	Experiments			
	Humoral activity		Melanization response	
Parameters	Group A	Group B	Group A	Group B
Wing length	2.70±0.020	2.75±0.022	2.88±0.021	2.78±0.011
No. of mosquitoes	20	20	20	20
p-value	>0.05	>0.05		

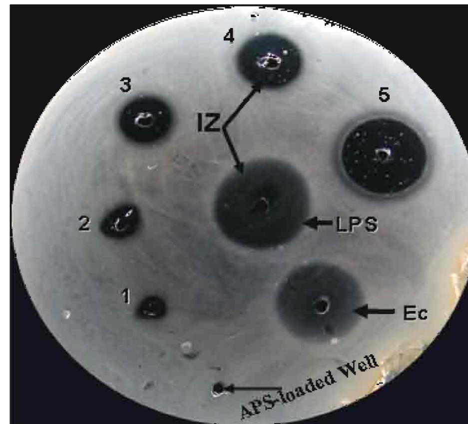

Fig. 3: An example of Petri dishes (9 cm; Sterilin) containing *M. luteus*-seeded agar representing inhibition zones, after incubation for overnight at 30ºC, showing the antibacterial activity of *Ae. aegypti*. At 24 h post-injection, fresh haemolymph from LPS-injected and *E. coli*-injected mosquitoes was loaded into wells [2 µL/well of diluted haemolymph in sterile anticoagulant II solution (Mead *et al*., 1986)]. Inhibition of bacterial growth appears as a clear inhibition zone (IZ). Zone diameter depends on the concentration of antibacterial peptides exist in the loaded haemolymph. Wells from 1-5 were loaded with 5 ascending concentrations of Lysozyme (1:100-1:16) as a standard (Ahmed, 2004). A negative control well (loaded with sterile APS was used in each dish. Diameters were measured directly using ruler and concentrations of peptides were calculated as Lysozyme equivalents from the resulting standard curve

Comparing to APS-injected mosquitoes, haemolymph from LPS-injected ones showed higher activity against *M. luteus* and *E. coli* (5.07 and 4.78 folds, respectively) than that against *B. subtilis* (2.4 folds) (Fig. 4-6). Haemolymph from *B. subtilis*-injected mosquitoes showed a steadily low activities against *B. subtilis*, *E. coli* and *M. luteus* (2.0, 1.7, 1.9 folds, respectively). Haemolymph from *E. coli*-injected mosquitoes showed a moderately higher activity against *M. luteus* (3.4 folds) compared to that against *B. subtilis* and *E. coli* (2.8 and 2.1, respectively) (Fig. 4-6). These findings may indicate that *M. luteus* is the most sensitive to the humoral activity of *Ae. aegypti* regardless the inducing agent and that the toxic bacteria, *B. subtilis* was less sensitive to the same activity.

Enhancive Effect of Thymoquenone Humoral Activity

The effect of Thq on the humoral anti-bacterial activity induced by the challenge with *B. subtilis* and *E. coli* against the same bacteria was demonstrated in this experiment as shown in Fig. 1. Haemolymph from *B. subtilis*-injected mosquitoes showed humoral activity against the same bacteria

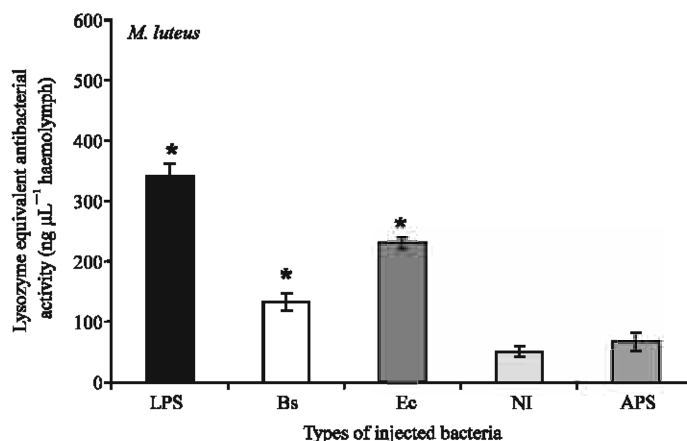

Fig. 4: The humoral antibacterial activity of blood-fed *Ae. aegypti* mosquitoes against *M. luteus* at 24 h post-injection with 20 ng LPS and challenging with *B. subtilis* (Bs) or *E. coli* (Ec). None-injected (NI) or sham injected (with APS) mosquitoes were used as controls. Haemolymph was collected 24 h post-injection and subjected to inhibition zone assay against *M. luteus.* Error bars represent standard errors of means of five replicates in each case (n = 5). Stars on columns represent significant higher activities compared to that of non-injected (NI) or APS- injected mosquitoes

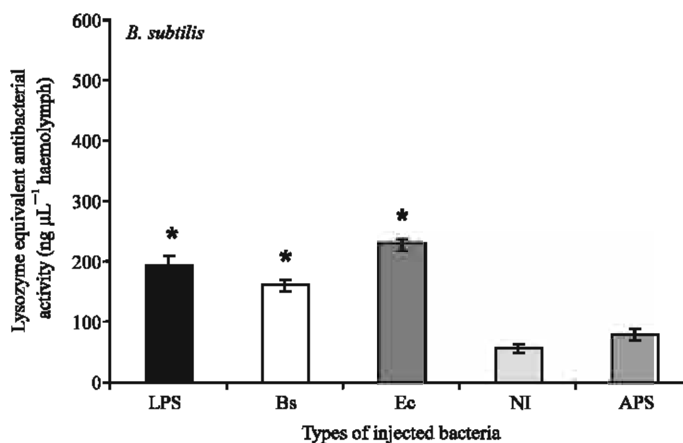

Fig. 5: The humoral antibacterial activity of blood-fed *Ae. aegypti* mosquitoes against *B. subtilis* at 24 h post-injection with 20 ng LPS and challenging with *B. subtilis* (Bs) or *E. coli* (Ec). None-injected (NI) or sham injected (with APS) mosquitoes were used as controls. Haemolymph was collected 24 h post-injection and subjected to inhibition zone assay against *B. subtilis.* Error bars represent standard errors of means of five replicates in each case (n = 5). Stars on columns represent significant higher activities compared to that of non-injected (NI) or APS-injected mosquitoes

which was equivalent to 574.6±14.4 ng μL^{-1} haemolymph. This activity was significantly higher than that of APS-injected mosquitoes (60.71±9.14 ng μL^{-1} haemolymph) by 9.5 folds ($p<0.05$) (Fig. 7). Thus, 0.3% Thq-glucose fed mosquitoes showed enhancing level of this activity by 4.7 folds against

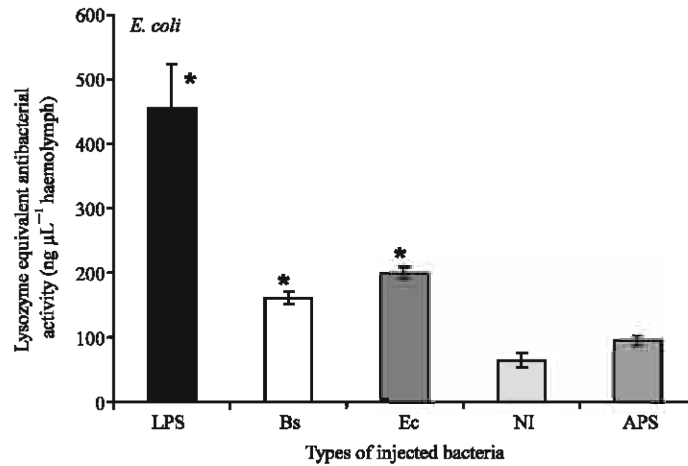

Fig. 6: The humoral antibacterial activity of blood-fed *Ae. aegypti* mosquitoes against *E. coli* at 24 h post-injection with 20 ng LPS and challenging with *B. subtilis* (Bs) or *E. coli* (Ec). None-injected (NI) or sham injected (with APS) mosquitoes were used as controls. Haemolymph was collected 24 h post-injection and subjected to inhibition zone assay against *E. coli*. Error bars represent standard errors of means of five replicates in each case (n = 5). Stars on columns represent significant higher activities compared to that of non-injected (NI) or APS-injected mosquitoes

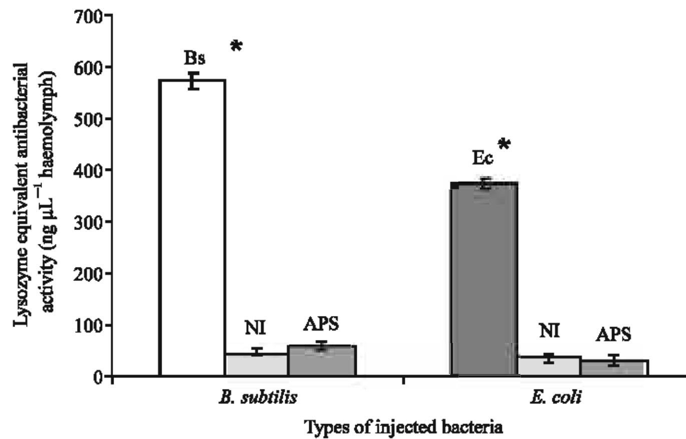

Fig. 7: Enhanced humoral antibacterial activity of blood-fed *Ae. aegypti* mosquitoes (maintained on 0.3% Thq-glucose solution) against challenged *B. subtilis* and *E. coli* bacteria at 24 h post-injection with the same bacteria. None-injected (NI) or sham injected (with APS) mosquitoes were used as controls. At 24 h post-injection, haemolymph from *B. subtilis*-injected (Bs) or *E. coli*-injected (Ec) mosquitoes was subjected to inhibition zone assay against *B. subtilis* or *E. coli* bacteria respectively. Haemolymph from non-injected (NI) or sham-injected (APS) mosquitoes was used as control for each case (n = 5). Error bars represent standard errors of means of five replicates in each case. Stars on columns represent significant higher activities compared to that of non-injected (NI) or APS- (sham) injected mosquitoes

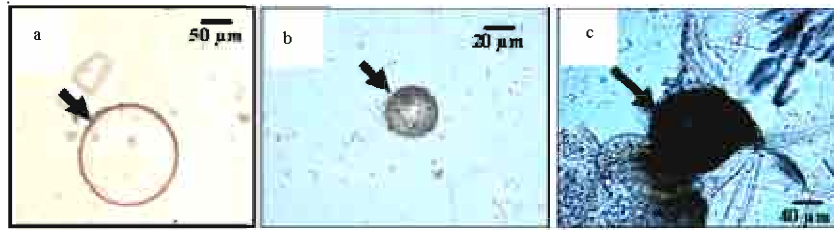

Fig. 8: Melanization degrees of Sephadex® beads by blood-fed *Ae. aegypti* mosquito. One bead, in 0.25 µL APS, was inoculated into mosquito thorax (one bead/mosquito). Thoraces were then dissected for monitoring melanized beads at 24 h post-inoculation. Based on the intensity of melanization, beads were categorized into three degrees; un-melanized or white (a), half melanized or patchy (b) and completely melanized or black (c) Arrows point at the target beads

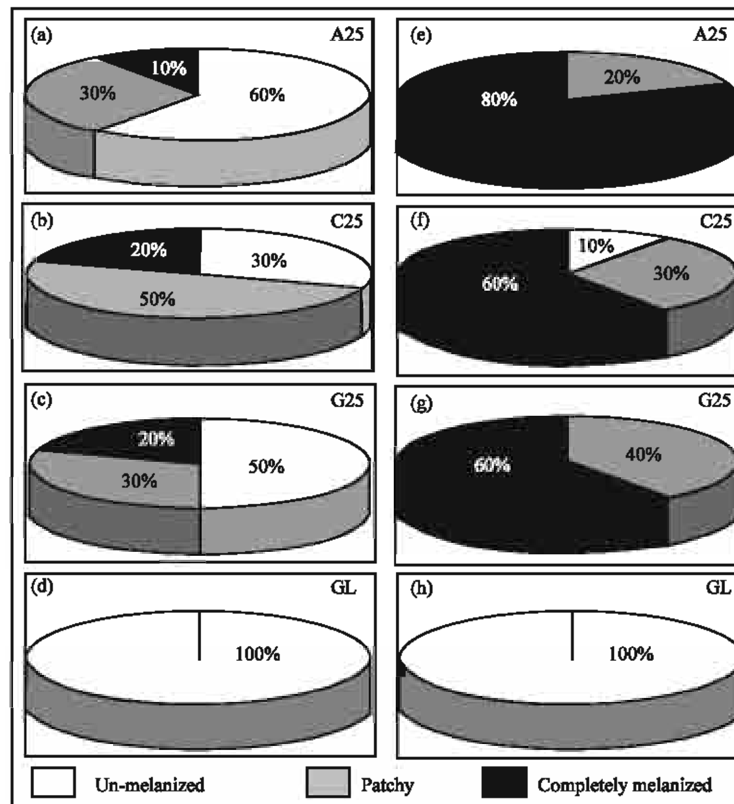

Fig. 9: Beads melanization by non-immune enhanced (fed on 10% glucose solution) (a-d) or immune enhanced (fed on 0.3% Thq-glucose mixture) (e-h) blood-fed *Ae. aegypti* mosquitoes. Different melanization degrees were monitored against the Sephadex® beads, CM A-25, CM C-25 and CM G-25 and the glass beads GL. Beads were inoculated into the thoraces of mosquitoes immediately after a blood meal. Thoraces from each mosquito groups were dissected at 24 h post-beads inoculation for scoring un-melanized (White), half melanized or patchy (shadow) and completely melanized (black) beads. Numbers shown on charts represents % of mosquitoes [mean % of 10 replicates (n = 10)] that showed each of the different melanization degrees

B. subtilis compared to that fed on 10% glucose solution only (2.0 folds of APS-injected mosquitoes) (Fig. 5). Moreover, haemolymph from *E. coli*-injected mosquitoes showed humoral activity against the same bacteria which was equivalent to 374±10.14 ng μL^{-1} haemolymph. This activity was significantly higher than that of APS-injected mosquitoes (30.73±10.1 ng μL^{-1} haemolymph) by 12 folds ($p<0.05$) (Fig. 7). Thus, 0.3% Thq-glucose fed mosquitoes showed enhancing this activity by 6 folds against *E. coli* when compared with that fed on 10% glucose solution only (2.1 folds of APS-injected mosquitoes) (Fig. 6). This may indicate an enhancive effect for the Thq on the humoral anti-bacterial activity and that this enhancive effect is dependant on the type of microbe.

Melanization Response and Effect of Thymoquenone

In this experiment, percentages of mosquitoes showed any of the three melanization degrees were calculated within 10% glucose fed or 0.3% Thq-glucose fed groups. In glucose fed groups, mosquitoes inoculated with A-25, C-25 or G-25 showed 60, 30 or 50% un-melanized (white) beads, respectively, 30, 50 or 30% half melanized beads respectively and 10, 20 or 20% fully melanized (black) beads, respectively (Fig. 8, 9a-c). However, 100% of mosquitoes showed un-melanized glass beads (Fig. 9d). On the other hand, 0.3% Thq-glucose fed groups, mosquitoes inoculated with A-25, C-25 or G-25 showed 0, 10 or 0% un-melanized (white) beads respectively, 20, 30 or 40% half melanized beads respectively and 80, 60 or 60% fully melanized (black) beads respectively (Fig. 9e-g). However, 100% of mosquitoes showed un-melanized glass beads (Fig. 9h). These data showed more melanized beads by Thq-fed mosquitoes compared to glucose-fed ones which may indicate that Thq has an enhancive effect on melanization response in *Ae. aegypti*. However, inert glass beads did not induce the melanization response in either cases, which may indicate that this immune response is charge-dependant.

DISCUSSION

In this study, it is important to clarify three points; a) melanization and humoral activity were proved to be the most effective immune responses against parasites and microbes in refractory mosquitoes (Collins *et al*., 1986; Shahabuddin *et al*., 1998; Hernandez *et al*., 2003; Angular *et al*., 2005) and thus, they were studied here against the Sephadex® beads model and live bacteria respectively as they might be the target responses to be utilized in the immuno-control strategy, b) dengue fever virus is being passed to mosquito via the blood meal which, on the other hand, induces a detectable level of humoral activity and enhances melanization (Ahmed, 2005a, b), thus, experiments of this study have been carried out on mosquitoes immediately after taking a blood meal and c) the size of the blood meal taken by mosquito may participate as a direct/indirect factor in the differences in melanization degrees and humoral activity (Koella and Sørensen, 2002) and therefore, blood meal size was accounted for to be certain that detected differences in this study were not because of different sized blood meals. Thus, wing size (the distance from the wing tip to the distal end of the allula) was used as an estimate of body size (Table 1) and hence, blood meal size (Briegel, 1990).

Black seed, *N. sativa*, a Dicotyledonous belongs to the botanical family of Ranunculaceae, has been employed as protective and curative agent for numerous human diseases and is known to have many treatment properties in traditional medicine (Chopra *et al*., 1956; Nadkarni, 1976). Thus, the biological effect of *N. sativa*, its oil and its active ingredients (quinones, thymoquenone and dithymoquinone) have been intensively studied pharmaceutically (Daba and Abdel-Rahman, 1998; Nagi *et al*., 1999). *N. sativa* has also been reported to have various antimicrobial activities (Worthen *et al*., 1998; Hanafy and Hatem, 1991). Recently, several studies have shown the enhancive effect of the black seed on the immune system of both vertebrates (Haq *et al*., 1999;

El-Dakhakhny *et al*., 2002; Abdel-Ghaffar *et al*., 2003) and insects (Abu El-Magd, 1995; Ahmed, 2004). In the current study, thymoquenone has shown an enhancive agent to the immune response of the dengue vector *Ae. aegypti*.

In the current study, mosquitoes (maintained on 10% glucose solution) showed significant induction in both humoral activity and melanization response against bacteria and Sephadex® beads respectively. On the other hand, mosquitoes, (maintained on 0.3% Thq-glucose mixture) showed significant increases in the humoral activity which were higher (up to 6 folds) and melanization response compared to that of glucose-fed mosquitoes. This, in fact, may indicate an enhancive effect for the Thq on immune response which could be in support of the immuno-control strategy of mosquito-borne diseases. These findings may be in support of Ahmed (2004) who recorded significant reduction in the number of mature oocysts in malaria-infected mosquitoes maintained on Black Seed Oil (BSO)-glucose mixture. This indicates that the reduction in oocysts number may have happened via BSO-induced (and probably thymoquenone in particular) immune response(s) that were sufficient to kill a significant number of developing ookinetes/young oocysts in the midgut. Furthermore, BSO induced immune stimulation may refer to one or all of its active constituents (quenones, thymoquenone and dithymoquenone) (Daba and Abdel-Rahman, 1998; Nagi *et al*., 1999). Data of the current study provide an evidence supporting that BSO may have enhanced immunity via its main active ingredient, the thymoquenone (Thq). And hence, these data suggest that if a viral-infective blood meal is synchronized with feeding mosquitoes on 0.3% of BSO or Thq, it may sufficiently induce immune responses to block dengue life cycle in mosquito midgut.

The mosquito*Ae. aegypti* transmits dengue viruses to humans when ingested during blood feeding and first encounters the midgut (Fig. 10). The virus then infects and replicates in the midgut epithelium, escapes the midgut and replicates at secondary sites within the body before ultimately infects the salivary glands (Fig. 10). Once the salivary glands are infected, the female mosquito is competent for transmission for the duration of her life. In order to overcome or, at least, to minimize the pathological effects, insects have developed different defence mechanisms that working in parallel against pathogens. One of these mechanisms is the innate immune response (Lowenberger, 2001), which includes the synthesis and secretion of antimicrobial peptides and degradation enzymes (Cheng *et al*., 2006), phagocytosis and melanization (Rivkin *et al*., 2006; Herrero *et al*., 2007) (Fig. 10). In this particular point, it has been proved that the predominant insect immune response against bacterial and fungal infections can also work against viruses (Sandersa *et al*., 2005; Brown and Hancock, 2006; Büyükgüzel *et al*., 2007). Based on these findings, genetic manipulation of the vector to become incompetent of transmitting dengue viruses has been raised up specially after establishing the vector genome (Severson *et al*., 2004) in order to profoundly and permanently reduce its vectorial competence and subsequently, block the transmission of dengue viruses to human. For example, Travanty *et al*. (2004) have identified RNA interference (RNAi) as a potential anti-viral, intracellular pathway in the vector that can be triggered by expression of virus-specific double stranded RNAs to reduce vector competence to dengue viruses and block the virus replication in its vector preventing transmission of the viruses. Thus, the recent real challenge is to utilize these strategies to silence virus replication in the vector (Fig. 10).

In conclusion, underlying the hypothesis of the approach of releasing genetically engineered pathogen-resistant mosquitoes, this will reduce or eliminate specific pathogen transmission in a target geographical area. The validation of this hypothesis has already been proved by developing transgenic *Ae. aegypti* mosquitoes which become resistant to the transmission of dengue viruses. Our data presented here may be in support of this immuno-control strategy that has the advantage of harnessing the naturally occurring vector responses to block the propagation of dengue virus inside its body and profoundly affect virus transmission. The current study showed a possibility to enhance the

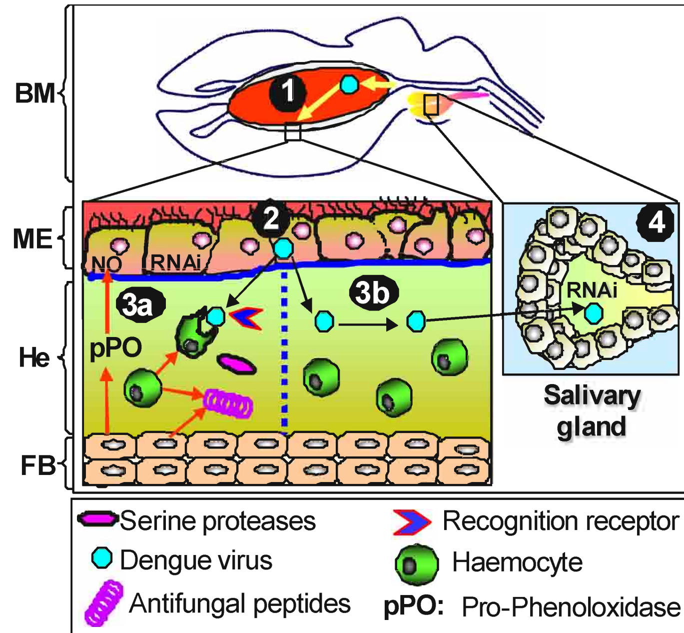

Fig. 10: A speculative illustration showing the dengue virus life cycle and the expected restriction factors/sites for its replication. When a mosquito takes a viremic blood meal (BM) (1), the virus encounters several barriers to infection. First, the virus must invade the midgut epithelium (ME) by overcoming a midgut infection barrier (2). In the midgut epithelium, the virus either succeeds to replicate or face an RNA-interference factor (RNAi) which blocks its replication, or the lyses factor, nitric oxide (NO) (Cao *et al*., 1998; Peterson *et al*., 2007). Having successfully replicated in the midgut epithelium, the virus must pass through a midgut escape barrier to the haemolymph (He) at which it either face another array of humoral or cellular response such as anti-microbial peptides, melanization [via prophenoloxidase (pPO)] cascade or phagocytosis (3a), or pass successfully (3b) to the salivary glands where its final residency as it undergoes more replication and waits for passing to a new vertebrate host. In the salivary glands (4), the virus may again face RNAi that may also restrict more viral replication, or being successfully transmitted to the next vertebrate host *via* saliva during the next mosquito blood feeding

antimicrobial responses of *Ae. aegypti* by more than 6 folds, which in turn, provides an evidence supporting the hope of utilizing the immune responses in the battle against dengue virus. Hence, whether the immune genes/responses of this dengue vector are induced upon viral infection and/or the antimicrobial peptides and enzymes, as well as cellular responses, can work efficiently against dengue virus is still to be investigated in the future study.

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