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Bacillus thuringiensis Induces Cellular Stress in the Mosquito Vector, *Culex pipiens*, Prior to Death

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Abstract.- This study was conducted to investigate the oxidative stress and apoptotic signs detectable by flow cytometry as proposed pathogenicity mechanisms for the mosquitocidal bacterium *Bacillus thuringiensis* (*Bt*) in the mosquito vector, *Culex pipiens*. Obtained data showed elevation in the levels of the oxidative stress biomarkers, the lipid peroxidation and protein oxidation, upon *Bt*-infection. Larvae showed significant higher levels of both lipid peroxidation and protein oxidation at 12 and 24h post-infection compared to control ones. In addition, *Bt*-inoculated adult mosquitoes also showed significant higher lipid peroxidation at 12 and 24h post-inoculation compared to control ones. These signs of oxidative stress were more pronounced in bacterial infected larvae than in bacterial inoculated adult mosquitoes. Finally, *Bt*-infected larvae showed significant higher percentages of cellular apoptosis at 12 an 24h post-infection compared to control ones. These data may indicate that *Bt* infection induced oxidative stress and apoptosis proceeding cellular damage, and thus, may be suggested as important pathogenicity mechanisms of *Bt* in its mosquito host. And hence, these data may participate in improving our understanding of the mosquito-*Bt* interaction scenario, which may help improving the biocontrol measurements against mosquito vectors.

Keywords: lipid peroxidation, oxidative protein products, apoptosis, *Bacillus thuringiensis*, *Culex pipiens*, oxidative stress.

INTRODUCTION

Insects are exposed to different challenging factors such as pathogens and contaminants in their breeding sites. Immune defense against pathogens is one of the crucial defense mechanisms that refer to antibacterial peptides and other immune-related molecules (Hultmark, 1998; Boman, 1998; Kavanagh and Reeves 2004; Imler and Bulet, 2005). These molecule, as well as cellular responses, kill invading microorganisms or parasites (Gillespie *et al.*, 1997; Morton *et al.*, 1987; Vilmos and Kurucz, 1998; Hultmark, 1998; Irving *et al.*, 2005; Wang *et al.*, 2011).

Reactive oxygen species (ROS) are also used by insect as cytotoxic materials against invading pathogens and parasites (Fang, 1999; Peterson and Luckhart, 2006). On the other hand, this ROS are radicals that can cause cellular toxicity like lipid peroxidation which disrupts membrane fluidity and the degradation products can initiate cellular apoptosis (Halliwell and Gutteridge, 1999; Kannan and Jain, 2000). Oxidative damage to proteins is another oxidative stress biomarker that can range from specific amino acid modifications and fragmentation of the peptide chain to total enzyme inactivation by superoxide anions (Stadtman, 1986). ROS can also lead to DNA damage *via* deletions, mutations, base degradation, single strand breakage and cross-linkage of proteins (Imlay and Linn, 1988; Imlay, 2003). Therefore, host protection against oxidative stress is vital for homeostasis and hence survival.

Insect host induces antioxidant defense by producing particular antioxidant enzymes that keep the balance between oxidants and antioxidants levels (Felton and Summer, 1995; Hyršl *et al.*, 2007). These antioxidant enzymes include SOD, CAT, GPx, GSTs and APOX (Ahmad, 1995; Halliwell and Gutteridge, 1999; Radyuk *et al.*, 2003). Transferrine and GSH are important antioxidants

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Abbreviations
AOPP, advanced oxidative protein products; APOX, ascrobate peroxidase; APS, *Aedes* physiological saline; *Btk*, *Bacillus thuringiensis kurstaki*; CAT, catalase; GPx, glutathione peroxidase; GSH, glutathione reduced form; GST, glutathione S transferase; LP, lipid peroxidation; LPS, lipopolysaccharide; MDA, malondialdehyde, NO, nitric oxide; PHGPx, phospholipid hydroperoxide glutathione peroxidases; PO, phenoloxidase Prxs, peroxiredoxins; ROS, reactive oxygen species; SOD, superoxide dismutase.

that help keeping this oxidative/antioxidative balance. The antioxidant controls the level of free irons (Yoshiga *et al.*, 1997) and has been shown to be up-regulated upon microbial infection (Valles and Pereira, 2005; Kim *et al.*, 2008). Hence, it has been suggested that a possible important role of insect transferrin could be in forming a general part of the insect immune response and functioning as an antibiotic against pathogens (Meister and Anderson, 1983) that allow insects to survive in a contaminated environment (Poupardina, *et al.*, 2008) and insecticide-resistance (Prapanthadara *et al.*, 1993). Beside playing a central role in the metabolism of insecticides and other xenobiotics (Feyereisen, 2005; Hemingway *et al.*, 2004), GSH constitutes a second line in insect immunity. It plays a role in the detoxification of toxins in insect body, including toxic immune compounds that involve melanin, and protecting insects from the concomitant oxidative stress (Nappi and Vass, 2001; Kumar *et al.*, 2003).

In previous studies, the antioxidative response, in terms of high GSH titer and its interaction with melanization immune response, in terms of phenoloxidase (PO) titer, have been investigated in mosquitoes upon infection with *B. thurengiensis* (Ahmed 2011, 2012). These studies showed suppression in antibacterial immune response in terms of low NO and PO titer and antioxidant defense in terms of low GSH titer upon infection of mosquitoes with *B. thuringiensis* (*Bt*). Therefore, it would be interesting to support these finding by investigating oxidative stress biomarkers and the concomitant cellular impact in *Bt*-infected mosquitoes. Thus, in the study proposed herein, lipid peroxidation and protein oxidation, as important biomarkers of oxidative stress, as well as cellular apoptosis, have been investigated upon *Bt*-infection in the mosquito vector, *Cx. pipiens*.

MATERIALS AND METHODS

Mosquito rearing

Culex pipiens mosquitoes were reared under standard insectarium conditions (26ºC, 12h/12h light/dark period and 80–82% humidity). Larvae were reared in tap water (or distilled water for experimental purposes) in the insectary of Zoology Department, College of Science, King Saud University, as previously detailed in Ahmed *et al.* (1999). Adults emerging within a 24h period were maintained in rearing cages (30 × 30 × 30 cm each) with continuous access to a 10% glucose solution (w/v). Both adult mosquitoes and larvae were used for the relevant experiments in this study. To maintain a stock of mosquito colony, they were kept accessing a blood meal frequently for triggering vitellogenesis (Clements, 1992).

Experimental bacteria

Mosquito-larvicidal bacterium, *Bacillus thuringiensis* (serotype H-3a & 3b, strain Z-52, Biotech International Ltd, India) was obtained from the Saudi Ministry of Agriculture as a spore-crystal powder [formulation contains 5-8% spores (w/w) and 5-8% delta endotoxins (w/w) and used based on the company's instructions]. For adult mosquito inoculation, *Bt* bacteria (spores from the spore-crystal powders) were routinely incubated in nutrient broth (13 g/l) at 37ºC for 48h in a rotary shaker at 200 rpm until a spectrophotometrical optical density OD_{598} of 0.5-0.7 is reached using UV Visible Spectrometer (Ultrospec, 2000, Pharmacia Biotech), then used for inoculating mosquitoes according to Nimmo *et al.* (1997).

Infecting larvae with bacteria

A rearing tray of one liter distilled water containing 100 third instar larvae of *Cx. pipiens* was left without treatment (control) or treated with LC_{50} (0.058mg/l) (Ahmed, 2011) of spore-crystal powders of *B. thuringiensis* (serotype H-3a and 3b, strain Z-52, Biotech International Ltd, India) based on the company's instructions. Larvae were allowed access to food (ground Goldfish Flake Food, Wardley®, USA: www.wordley.com) throughout the experiments. Infected or non-infected (control) larvae were used for experimental analysis as detailed below.

Inoculating adult mosquitoes with bacteria

Bacterial suspensions of *Bt* (OD_{598} of 0.5-0.7) were prepared as detailed above. Fifty adult mosquitoes (6-days old) were immobilized, by chilling on ice for 5 min, prior to inoculation (piercing) with a hand made glass capillary needle. Ice-chilled mosquitoes were pierced through a

lateral side of the thoracic cavity, deeply enough to penetrate the layer of fat body beneath the epidermis, but at an angle oblique enough to avoid piercing the gut. Piercing needle was pre-dipped in *Aedes* physiological saline (APS) (13 mM NaCl, 0.5 mM KCl, 0.1 mM $CaCl_2$) as trauma control, or *Bt* bacteria according to Dimopoulos *et al*. (1997) and Ahmed (2011). Any mosquito that was severely bled after inoculation was discarded from the study. Mosquitoes were then allowed to recover and maintained in appropriate cages (16 × 16 × 16 cm each) under the usual standard rearing in insectarium conditions. Mosquitoes were then used for biochemical assays at 12 and 24h post-inoculation. Only active mosquitoes (able to fly) were used for experimental purposes. Five independent replicates (from five different individual mosquitoes) (n=5) were carried out in each experiment to perform statistical analysis.

Determining lipid peroxidation (LP)

Body homogenates from adult mosquitoes or larvae were used for estimating endogenous lipid peroxidation spectrophotometrically following the method described by Okhawa *et al.* (1979) expressed in a nano-moles of malondialdehyde (MDA) per milliliter homogenate (nmole/ml). Fifty control or bacterial-infected 3rd instar larvae (with LC_{50} = 0.058mg/l) or bacterial inoculated adult mosquitoes were homogenized in 500 ml of phosphate buffer (0.05 M, pH 7.2) containing 2mM ethylenediaminetetraacetic acid (EDTA, Sigma), 0.5mM dithiothreitol (DTT, Fluka), 0.8mM phenylmethylsulphonyl fluoride (PMSF, Sigma) and 1.5% polyvinylpyrolidone (PVP, Sigma). Homogenates (from larvae or adult mosquitoes) were then centrifuged at 16,000g at 4ºC for 30 min. An amount of 0.5 ml of the resulting supernatant was shaken with 2.5 ml of 20% trichloroacetic acid (TCA). One ml of 0.67% thiobarbituric acid (TBA) was added to the resulting mixture, shaken, and warmed up for 30 min in a water bath, followed by immediate rapid cooling in ice for 5 min. After cooling, 4 ml of n-butyl-alcohol was added and shaken well. The resulting mixture was then centrifuged at 16,000g for 5 min. The resultant n-butyl-alcohol layer was moved into a separate tube and MDA content was determined spectrophotometrically at 535 nm using UV visible Spectrometer (Ultrospec 2000, Pharmacia Biotech).

Determining advanced oxidative protein products (AOPP)

Protein oxidation was spectrophotometrically measured by determining the AOPP levels in larval or adult mosquito homogenates at 12 or 24h post-treatment (Rugale *et al.*, 2007; Aiassa *et al.*, 2011). One milliliter from adult or larval homogenate (diluted 1/5 in PBS) was analyzed with 0.1 ml of glacial acetic acid and 50 µl of 1.16 M potassium iodide. One milliliter of 0–100 µM chloramine-T was used as standard (Correa Salde and Albesa, 2009). The absorbance of the reaction mixture was read at 340 nm using an UV Visible Spectrometer (Ultrospec 2000, Pharmacia Biotech) to calculate the chloramine-T equivalents protein products in µM/ml.

Determining cellular apoptosis

Apoptosis in control or infected 3rd larval instar was investigated using flow cytometry (BD FACSCalibur, US) according to the manufacturer instructions. Briefly, larval cells were manually isolated from control or *Bt*-infected larvae (at 8, 12, 16, 20 and 24h post-treatment) by gentle homogenization of larvae at each time point (20 larvae each) in 10 ml mixture of PBS and 0.04% EDTA. Larval homogenates were then filtered through a suitable fine-meshes refinery to collect single cells. Resulting cellular suspensions were then centrifuged for 30 min at 3000g at 4ºC. Supernatants were removed and cellular pellets were washed twice in PBS-EDTA buffer. Cellular pellets were then fixed and permeabilized by incubation in 70% ice-cooled ethanol for overnight. Cells were then washed twice in PBS-EDTA buffer and incubated at 37°C for 1 h in a mixture of 40 µg/ml propidium iodide and 100 µg/ml DNase free RNase in PBS-EDTA buffer for staining DNA. Samples were then analyzed by assessing FL2 red fluorescence on a linear scale using flow cytometry. The resulting percentages of apoptotic cells were determined as the percentages of hypodiploid cells (sub G0/G1 peaks).

Statistical analysis

All statistical analyses were undertaken using MINITAB software (MINITAB, State College, PA, v: 13.1, 2001). Data were first tested for normality (using Anderson-Darling test) and for variances homogeneity prior to any further analysis. Data pertaining to the LP, AOPP and apoptosis were normally distributed (Anderson Darling test) and thus, a two-sample t-test (for individual comparison) was used for comparing differences between treated and control larvae in each case. Five replicates (five different mosquito groups in each case: n = 5) were carried out for better statistical analysis.

RESULTS

The current study investigates the possibility that oxidants could induce stress resulting in damaging cellular lipid and protein molecules as well as cellular abnormalities in *Cx. pipiens* upon infection with *Bt*. Thus, this study focused mainly on lipid peroxidation, protein oxidation and apoptosis as indicative biomarkers of cellular collapse upon *Bt* infection in this mosquito vector.

Lipid peroxidation (LP) in larval mosquitoes

Larvae of *Cx. pipiens* were exposed to LC_{50} (0.058 mg/l) of *Bt* for 24 or 48h. Data from LP assay showed significant higher MDA concentration in the homogenate of *Bt*-infected larvae compared to that of control ones at 12h post-treatment (12.13±0.83 *v* 7.06±0.28 nmole/ml respectively) ($P < 0.05$, n=5, student t-test) (Fig. 1). For investigating long-term oxidative stress upon inoculation with *Bt*, MDA levels were measured at 24h post-inoculation in comparison with control mosquitoes. Data were still showing significant higher MDA concentration in *Bt*-infected larvae compared to that of control ones (11.64±0.64 *v* 5.26±0.35 nmole/ml respectively) ($P<0.05$, n=5, student t-test) (Fig. 1). This may indicate that *Bt* infection has significantly increased lipid peroxidation in terms of high level of malondialdehyde in the 3rd larval instar of *Cx. pipiens* up to 24h.

Lipid peroxidation (LP) in adult mosquitoes

Six-days old *Cx. pipiens* mosquito were inoculated with *Bt* prior to LP bioassay in terms of malondialdehyde (MDA) titer. Data from LP assay showed significant higher MDA concentration in the homogenate of mosquitoes at 12h post-inoculation with *Bt* bacteria compared to that of APS-inoculated (control) ones (2.33±0.23 *v* 1.00±0.23 nmole/ml, respectively) ($P<0.05$, n = 5, student t-test) (Fig. 2). For investigating long-term oxidative stress upon inoculation with *Bt*, MDA levels were measured at 24h post-inoculation with *Bt* in comparison with control mosquitoes. Data were still showing significant higher MDA concentration in of mosquitoes homogenate at 24h post-inoculation with *Bt* bacteria compared to that of APS-inoculated (control) ones (7.07±0.82 *v* 1.66±0.38 nmole/ml respectively) ($P < 0.05$, n = 5, student t-test) (Fig. 2). This may indicate that *Bt* inoculation has a long-term adverse oxidative effect, in terms of lipid peroxidation, on adult mosquitoes.

Advanced oxidative protein products (AOPP) in larval mosquitoes

The oxidative effect of *Bt* infection on total proteins in the 3rd larval instar was investigated in this experiment. Data showed that *Bt*-infected larvae induced a significant increase of AOPP compared to that of control larvae (47.31±1.21 *v* 35.29±1.20 µmole/ml respectively) ($P < 0.05$, n = 5, student t-test) at 12h post-infection (Fig. 3). This significant increase in AOPP was also monitored at 24h post-infection compared to that of control larvae (88.62±2.21 *v* 42.77±2.18 µmole/ml respectively) ($P < 0.05$, n = 5, student t-test) (Fig. 3). This may indicate that *Bt* infection induces AOPP in infected larvae, which is a biomarker of protein damage.

Advanced oxidative protein products (AOPP) in adult mosquitoes

Protein damage, in terms of advanced oxidative protein products (AOPP), was also investigated in 6-days old *Cx. pipiens* mosquito upon inoculation with *Bt*. Data from bacteria-inoculated mosquito homogenates showed significant higher AOPP concentration at 12h post-inoculation compared to that of APS-inoculated (control) ones (29.66±2.49 *v* 15.00±1.98 µmole/ml respectively) ($P < 0.05$, n = 5, student t-test) (Fig.4). For investigating long-term protein damage in adult

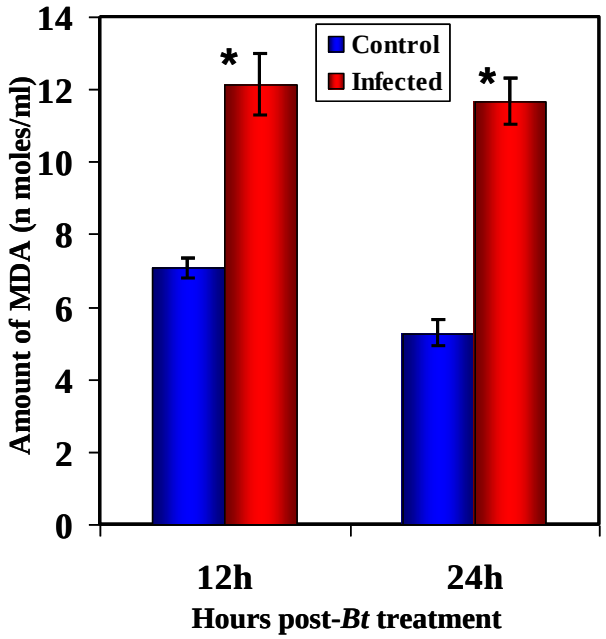

Fig. 1. Malondialdehyde (MDA) titer (nmole/ml) in the 3rd larval instars of the *Cx. pipiens* mosquito. Larvae were treated with LC_{50} (0.058 mg/l) of *B. thuringiensis* spore-crystal powder or left without treatment (control). MDA titer was measured spectrophotometrically (at 535 nm) at 12 and 24h post-*Bt* infection. Error bars represent standard errors of means of 5 replicates each (N = 5). Asterisk (*) represents significant higher MDA level comparing to control larvae ($P < 0.05$, two-sample t-test).

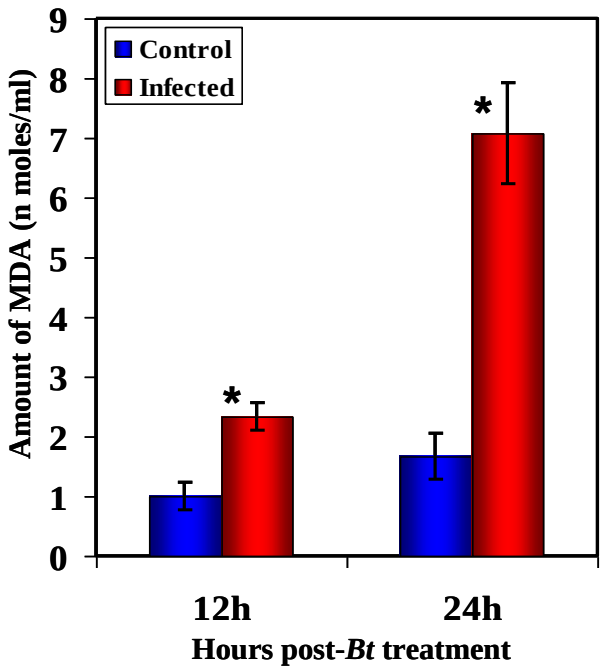

Fig. 2. Malondialdehyde (MDA) titer (nmole/ml) in 6-days old *Cx. pipiens* mosquitoes post-inoculation with *B. thuringiensis*. In inoculated mosquitoes, MDA concentration was measured spectrophotometrically (at 535 nm) at 12 and 24h post-inoculation. Error bars represent standard errors of means of 5 replicates each (N = 5). Asterisk (*) represents significant higher MDA level comparing to control mosquitoes ($P < 0.05$, two-sample t-test).

mosquitoes upon inoculation with *Bt*, AOPP level was measured at 24h post-inoculation with *Bt*. Data showed higher AOPP concentration in the homogenate of *Bt*-inoculated mosquitoes at 24h post-inoculation compared to APS-inoculated (control) ones (48.28±3.39 *v* 18.41±2.41 µmole/ml respectively) ($P>0.05$, n = 5, student t-test) (Fig. 4). This may indicate that *Bt* inoculation induces AOPP in adult *Cx. pipiens*, which is a biomarker of protein damage.

Cellular apoptosis

Flow cytometry was used in this experiment to monitor the population homogeneity of whole larval cells *via* side and forward scatters parameters (Fig. 5A). The percentages of apoptotic cells in control or infected larvae were determined as the percentages of gated cells located in the sub G1 histograms. As shown in Figure 5B and C, cells isolated from control and *Bt*-infected larvae (at 12h post-infection), respectively, represent low percentage of apoptosis (3.05% and 4.63%, respectively) (Table I). However, the percentage of apoptotic cells in infected larvae at this time point of experiment was significantly higher than that of control one ($P < 0.05$, N = 5, student t-test). Moreover, cells isolated from *Bt*-infected larvae at 24h-post-infection represented significant elevated apoptotic population (41.93%) as shown in figure 5D and Table I. ($P < 0.05$, N = 5, student t-test).

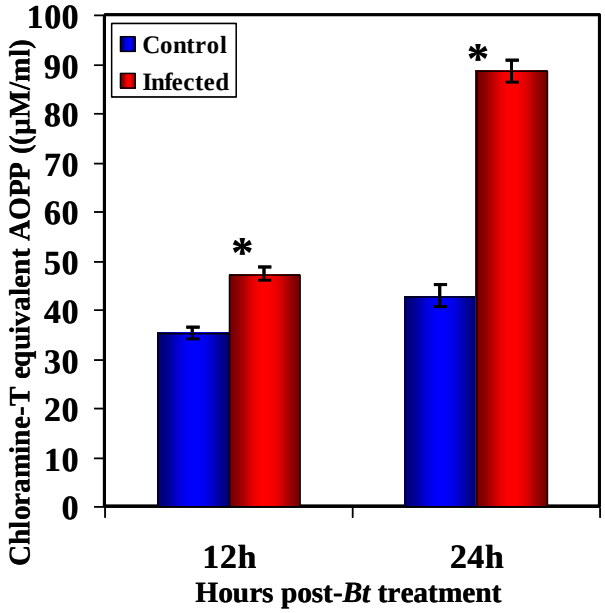

Fig. 3. Oxidation of proteins to AOPP in the 3rd larval instar of autogenous *Cx. pipiens* mosquitoes at 12 and 24h post-infection with *Bt*. AOPP concentration was measured spectrophotometrically (at 340 nm). Error bars represent standard errors of means of 5 replicates each (N = 5). Asterisk (*) represents significant higher AOPP comparing to control mosquitoes ($P < 0.05$, two-sample t-test).

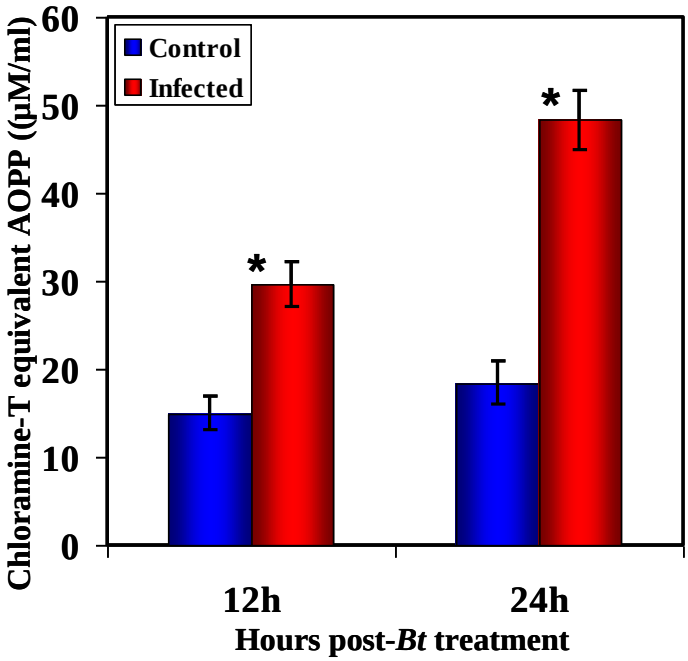

Fig. 4. Oxidation of proteins to AOPP in 6-days old autogenous *Cx. pipiens* mosquitoes post-inoculation with *Bt*. In inoculated mosquitoes, AOPP concentration was measured spectrophotometrically (at 340 nm) at 12 and 24h post-inoculation. Error bars represent standard errors of means of 5 replicates each (N = 5). Asterisk (*) represents significant higher AOPP level comparing to control mosquitoes ($P < 0.05$, two-sample t-test).

Table I.- Percentages of cells undergoing apoptosis in control and *Bt*-infected 3rd instar larvae of the *Cx. pipiens* mosquito at 12 and 24h post-infection as detected by flow cytometry.

Parameters	Hours post-infection		
	Control (n=5)	12h (n=5)	24h (n=5)
Total no of tested cells	22404±114	22301±26	21764±119
Number of apoptotic cells	683.3±49.1	1043±30	9128±354
% of apoptotic cells	3.050±0.221	4.639±0.14*	41.93±1.41**

Mean±SEM.
* Significantly higher compared to control ($P < 0.05$, student t-test).
** Significantly higher compared to that at 12h or control ($P < 0.05$, student t-test).

DISCUSSION

In the current study, lipid peroxidation and advanced oxidation protein products are investigated as important biomarkers for oxidative stress in both larval and adult stages of the *Cx. pipiens*. Bioassays were carried out in adult mosquitoes (6 days-old) or 3rd larval instars at 12 and 24h post-*Bt* infection or inoculation respectively. Basically, lipid peroxidation is a chain reaction between polyunsaturated fatty acids and ROS, and it produces lipid peroxides and hydrocarbon polymers that are both highly cytotoxic (Horton and Fairhurst, 1987), and hence, increase oxidative stress. It has been proved that ROS cause lipid peroxidation, protein carbonylation, DNA oxidation and glutathione depletion, leading to oxidative damage

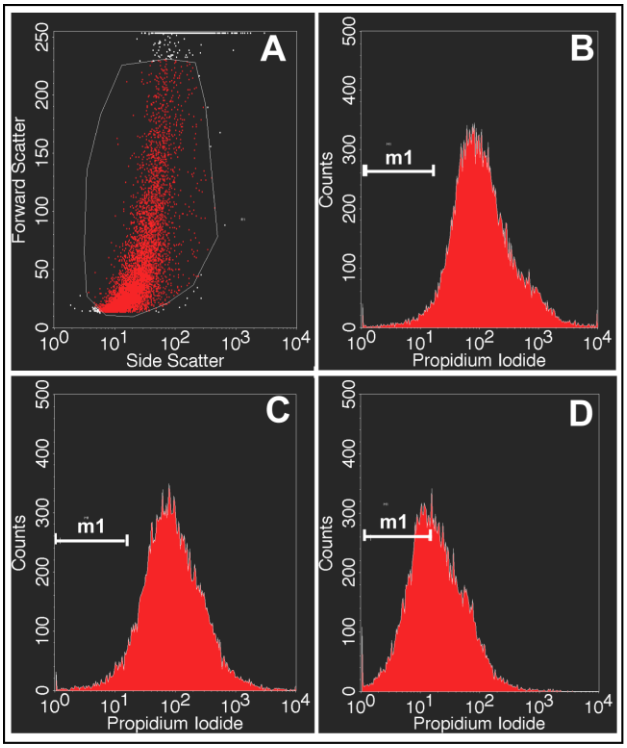

Fig. 5. Flow cytometric analysis enrolled to determine the percentage of cells undergoing apoptosis in control and *Bt*-infected 3rd instar larvae of *Cx. pipiens*. Apoptosis was determined as the percentage of hypodiploid cells. A, represents dot blot showing the homogenous gated cells population used for analysis. Histograms represent the staining with propidium iodide in control (B), at 12h post-infection (C) and at 24h post-infection (D). m1: represents mean number of cells in sub G1 stage (apoptotic).

and alterations in radical scavenging enzymes in insect tissues (Ahmad, 1995). The current study reported significant increase in TBA-reactive malonyldialdehyde at 12 and 24h respectively post infection with *Bt* in mosquito larvae as compared to un-infected controls. This may indicate high lipid peroxidation (Jain, 1984) that may have lead to cytotoxicity in infected larvae. In mammals and insects, phospholipid hydroperoxide glutathione peroxidases (PHGPx) has been shown to prevent lipid peroxidation and protect biomembranes against oxidative stress (Ursini *et al.*, 1982; Li *et al.*, 2003). This is because PHGPx are antioxidant enzymes that can directly reduce peroxidized phospholipids and cholesterols within membranes (Ursini *et al.*, 1985; Thomas *et al.*, 1990). Based on these studies, the observed significant increase of lipid peroxidation upon *Bt*-infection in the current study may suggest that *Bt* suppresses/deactivates the production of PHGPx in infected mosquito. Furthermore, *Bt* has been shown to have suppressive effect on both antioxidant and antibacterial immune activity in *Bt*-infected larvae (Ahmed, 2011). Taken all together, it would be reliable to suggest that these suppressive effects could be considered as pathogenicity biomarkers of *Bt* against mosquitoes.

Furthermore, exposure of cellular proteins to ROS results in oxidation of side-chains and fragmentation of the peptide chains (Davies and Dean, 1997). These ultimately results in the formation of AOPP, loss of structural or enzymatic activity of the protein and, hence, cytological dysfunction and finally biological interruption (Davies and Dean, 1997; Dean *et al.*, 1997; Capeille're-Blandin *et al.*, 2004; Descamps-Latscha *et al.*, 2005). This may have happened in *Bt*-infected larvae and *Bt*-inoculated adults of the current study as they showed significant increase in AOPP at 12 and 24h post-infection respectively compared to control ones. In insects, bacterial challenge normally activates upstream proteinases in the cascade, which activate prophenoloxidase (pPO) (Kanost and Gorman, 2008) leading ultimately to the formation of the cytotoxic melanin (Sugumaran *et al.*, 2000; Cerenius and Soderhall, 2004). This melanin cytotoxicity is attributed, in part, to its ability to bind covalently to cell-membrane components and other cellular nucleophiles. This subsequently promotes free-radical cascades and involves sulfydryl oxidations, inactivation of DNA polymerase, depolymerization of lipids and lipid peroxidation (Nappi and Vass, 2001; Christensen *et al.*, 2005). On the other hand, mosquitoes adapted a mechanism that protects their cells/tissues from this immune-related oxidative stressor *via* the antioxidant GSH (Erden-Inal *et al.*, 2002 and reviewed by Christensen *et al.*, 2005). Thus, the low GSH titer in *Bt*-infected mosquitoes that was observed by Ahmed (2011) explains the elevated level of AOPP recorded in *Bt*-infected mosquitoes in the current study.

In fact, ROS involvement in immune responses against foreign invaders have been proved by Herrera-Ortiz *et al.* (2011). It has been investigated that H_2O_2 and Nitric oxide NO are induced as anti-malarial immune response in the malaria-refractory mosquitoes (Kumar *et al.*, 2003; Herrera-Ortíz *et al.*, 2004, 2011; Peterson *et al.*, 2007) and other microbial-infected insect models (Imamura *et al.*, 2002; Foley and O'Farell, 2003). Mosquitoes, on the other hand, protect themselves against this immune-induced oxidative stresses through antioxidant defensive mechanism for their survival by minimizing the resulting cellular damage *via* peroxiredoxins (Prxs), enzymes known to detoxify ROS, as antioxidative response (Peterson and Luckhart, 2006, Townsend, 2007; Chen *et al.*, 2011). Data of the current study shows clearly that *Bt* has induced oxidative stress in infected mosquitoes. This may further explain the recorded low level of glutathione in *Bt*-infected mosquito larvae reported by Ahmed (2011) as a suppressive mechanism imposed by *Bt* bacteria which, subsequently, interrupted the antioxidant-oxidant balance in infected mosquitoes. And hence, this *Bt*-induced oxidative stress may be considered as a mechanism behind high pathogenicity of *Bt* against mosquitoes. Finally, the presented data data of the current study demonstrated that *Bt* infection induced oxidative stress in *Cx. pipiens* producing higher levels of ROS and this oxidative imbalance plays an important role in the larvicidal effect of this bacteria as shown for other toxins (Wei *et al.*, 2010; Beckon *et al.*, 2008). Furthermore, *Bt* might have interrupted the self-protection antioxidant system, and hence, exposed mosquito cells to the cell damaging oxidative stress leading to the reported cellular apoptosis.

In fact, apoptosis plays a key role in the development, regulation and function of the immune system, and can be a response of cells to general stress, including the stress incurred as a result of immune induction and infection with intracellular pathogens (Benedict *et al*., 2002; Teodoro and Branton, 1997; Ahmed and Hurd, 2006; Rost-Roszkowskaa *et al*., 2010; Roekring and Smith, 2010; Zhao *et al*., 2011). Thus, the significant higher percentage of apoptosis recorded in the current study could be attributed to the *Bt*-infection oxidative stress that triggered the apoptotic cascade through the activation of caspases, the family of cysteine proteases (Raff, 1998; Kumar and Doumanis, 2000; Richardson and Kumar, 2002; Kornbluth and White, 2005; Coopera *et al*., 2007). Flow cytometry detected apoptosis as early as 12h post-*Bt*-infection, which may imply that apoptosis proceeds cellular damage in the larval mosquito gut.

In conclusion, increased lipid peroxidation and protein oxidation shown in this study may be due to a) increased production of endogenous or exogenous pro-oxidants such as NO, xanthine oxidase, homocysteine (Stohs and Bagchi, 1995), b) deficiencies of antioxidants (ceruloplasmin, transferrin, SOD, GPx, CAT, GSH) or (c) both. This increased oxidative stress may have lead to cytotoxicity which ends up with cellular apoptosis and hence cytological damage (Kannan and Jain, 2000). Thus, suppression of antioxidants leading to elevation of ROS resulting in oxidative stress in terms of membrane lipid abnormalities, mitochondrial dysfunction, excitotoxicity and apoptosis (cellular damage), and innate immunity dysregulation in parallel (Ahmed, 2011) is the suggested scenario that may have happened in *Bt*-infected larvae of the current study prior to death. Therefore, I would suggest that the cytopathological effects of *Bt* in larval gut epithelial cells (Al-Roba1 *et al.*, 2011) may have happened *via* this suggested scenario. Finally, I would suggest that inhibition of ROS (through oral feeding of antioxidants) may reduce cytotoxicity (pathogenecity) of entomopathogenic microorganisms against beneficial insects and/or activation of endogenous ROS *via* adding exogenous antioxidant-inhibitors to the formula of biocontrol agents could be in the favor of biocontrol measures. Although trials of activating the antioxidant defense has been recently took place (Alquicer *et al.*, 2009), this exciting suggestion awaits to be proven.

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REFERENCES

AHMED, A.M., 2011. Immune and antioxidant defenses in an autogenous *Aedes caspius* mosquito upon infection with *Bacillus thuringiensis kurstaki*. *Afr. J. Microbiol. Res.*, **5**: 3848-3857.

AHMED, A.M., 2012. Lipid peroxidation and oxidative protein products as biomarkers of oxidative stress in the autogenous mosquito, *Aedes caspius*, upon infection with the mosquitocidal bacteria, *Bacillus thuringiensis kurstaki*. *Pakistan J. Zool.*, **44**: 525-536.

AHMAD, S., 1995. Oxidative stress from environmental pollutants. *Arch. Insect Biochem. Physiol.*, **29**: 135–157.

AHMED, A.M. AND HURD, H., 2006. Immune stimulation and malaria infection impose reproductive costs in *Anopheles gambiae* via follicular apoptosis. *Microb. Infect.*, **8:** 308–315.

AHMED, A.M., TAYLOR, P., MAINGON, R. AND HURD, H., 1999. The effect of *Plasmodium yoelii nigeriensis* on the reproductive fitness of *Anopheles gambiae*. *Inverteb. Reprod. Develop*., **36**: 217-222.

AIASSA, V., BARONETTI, J.L., PAEZ, P. L., BARNES, A.I., ALBRECHT, C., PELLARIN, G., ERASO, A.J. AND ALBESA, I., 2011. Increased advanced oxidation of protein products and enhanced total antioxidant capacity in plasma by action of toxins of *Escherichia coli* STEC. *Toxicology in Vitro*., **25**: 426–431.

AL-ROBA, A.A., ABOUL-SOUD, M.A.M., AHMED, A.M. AND AL-KHEDHAIRY, A.A., 2011. The gene expression of caspasses is up-regulated during the signaling response of *Aedes caspius* against larvicidal bacteria. *Afr. J. Biotechnol*., **10**: 225-233.

ALQUICER, G., KODRÍK, D., KRISHNAN, N., VEČEŘA, J. AND SOCHA, R., 2009. Activation of insect anti-oxidative mechanisms by mammalian glucagon. *Comp. Biochem. Physiol.*, Part B **152**: 226–233.

BECKON, W.N., PARKINS, C., MAXIMOVICH, A. AND BECKON, A.V., 2008. A general approach to modeling biphasic relationships. *Environ. Sci. Technol*., **42**: 1308–1314.

BENEDICT, C.A., NORRIS, P.S. AND WARE C.F., 2002. To kill or be killed: viral evasion of apoptosis. *Nature Immunol.*, **3**: 1013–1018.

BOMAN, H.G., 1998. Gene-encoded peptide antibiotics and the concept of innate immunity: an update review. *Scand. J. Immunol.*, **48**: 15–25.

CAPEILLE'RE-BLANDIN, C., VALE'RIE, G., DESCAMPS-LATSCH, B. AND WITKO-SARSAT, V., 2004. Biochemical and spectrophotometric significance of advanced oxidized protein products. *Biochim. biophys. Acta*, **1689**: 91–102.

CERENIUS, L. AND SODERHALL, K., 2004. The prophenoloxidase-activating system in invertebrates. *Immunol. Rev*., **198**: 116–126.

CHAUHAN, A. AND CHAUHAN, V., 2006. Oxidative stress in autism. *Pathophysiology*, **13**: 171–181.

CHEN, T.-H., TANG, P., YANG, C.-F., KAO, L.-H., LO, Y.-P., CHUANG, C.-K.., SHIH, Y.-T. AND CHEN, W.-J., 2011. Antioxidant defense is one of the mechanisms by which mosquito cells survive dengue 2 viral infection. *Virology*, **410**: 410–417.

CHRISTENSEN, B. M., LI. J., CHEN. CHENG-C. AND NAPPI, A.J., 2005. Melanization immune responses in mosquito vectors. *Trends Parasitol.*, **21**: 193-199.

COOPERA, D.M., PIOB, F., THIA, E.P., THEILMANNC, D. AND LOWENBERGERA, C., 2007. Characterization of *Aedes* Dredd: A novel initiator caspase from the yellow fever mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol*., **37**: 559–569

CLEMENTS, A.N., 1992. Vitellogenesis, In: *The biology of mosquitoes*, V.1. Cambridge University Press, pp. 360-379.

CORREA SALDE, V. AND ALBESA, I., 2009. Reactive oxidant species and oxidation of protein and haemoglobin as biomarkers of susceptibility to stress caused by chloramphenicol. *Biomed. Pharmacother*., **63**: 100–104.

DAVIES, M.J. AND DEAN, R.T., 1997. *Radical-mediated protein oxidation: from chemistry to medicine*. Oxford Univ. Press, Oxford, U.K.

DEAN, R.T.S., FU, R. AND DAVIES, M.J., 1997. Biochemistry and pathology of radical-mediated protein oxidation. *Biochem. J*., **324**: **1-8**.

DESCAMPS-LATSCHA, B., WITKO-SARSAT, V., NGUYEN-KHOA, T., NGUYEN, A.T., GAUSSON, V., MOTHU, N., LONDON, G.M. AND JUNGERS, P., 2005. Advanced oxidation protein products as risk factors for atherosclerotic cardiovascular events in nondiabetic predialysis patients. *Am. J. Kidney Dis*., **45**: 39–47

DIMOPOULOS, G., RICHMAN, A., MULLER, H-M. AND KAFATOS, F.C., 1997. Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Proc. natl. Acad. Sci. U.S.A*, **94**: 11508-11513.

ERDEN-INAL, M., SUNAL, E. AND KANBAK, E.G., 2002. Age-related changes in the glutathione redox system. *Cell. Biochem. Funct*., **20**: 61–66.

FANG, F.C. (Ed.), 1999. *Nitric oxide and infection*. Kluwer Academic/Plenum, New York.

FELTON, G.W. AND SUMMERS, C.B., 1995. Antioxidant systems in insects. *Arch. Insect Biochem. Physiol*., **9**: 187–197.

FEYEREISEN, R., 2005. Insect cytochrome P450. In: *Comprehensive molecular insect science* (eds. L. I. Gilbert, K. Iatrou and S. Gill), vol. 4. Elsevier, Oxford,

pp. 1–77.

FOLEY, E. AND O'FARELL, P.H., **2003**. Nitric oxide contributes to induction of innate immune responses to gram-negative bacteria in *Drosophila*. *Genes Dev*., **17**: 115–125.

GILLESPIE, J.P., KANOST, M.R. AND TRENCZEK, T., 1997. Biological mediators of insect immunity. *Annu. Rev. Ent*., **42**: 611–643.

HALLIWELL, B. AND GUTTERIDGE, J. M. C., 1999. *Free radicals in biology and medicine*. Oxford University Press, New York.

HEMINGWAY, J., HAWKES, N.J., MCCARROLL, L. AND RANSON, H., 2004. The molecular basis of insecticide resistance in mosquitoes. *Insect Biochem. mol. Biol*., **34**: 653–665.

HERRERA-ORTÍZ, A., LANZ-MENDOZA, H., MARTÍNEZ-BARNETCHE, J., HERNÁNDEZ-MARTÍNEZ, S., VILLARREAL-TREVIÑO, C., AGUILAR-MARCELINO, L. AND RODRÍGUEZ, M.H., 2004. *Plasmodium berghei* ookinetes induce nitric oxide production in *Anopheles pseudopunctipennis* midguts cultured *in vitro*. *Insect Biochem. mol. Biol*., **34**: 893–901.

HERRERA-ORTIZ, A., MARTÍNEZ-BARNETCHE, J., SMIT, N., RODRIGUEZ, M.H. AND LANZ-MENDOZA, H., 2011. The effect of nitric oxide and hydrogen peroxide in the activation of the systemic immune response of *Anopheles albimanus* infected with *Plasmodium berghei*. *Dev. Comp. Immunol.*, **35**: 44–50.

HORTON, A.A. AND FAIRHURST, S., 1987. Lipid peroxidation and mechanisms of toxicity. *CRC Crit. Rev. Toxicol*., **18**: 27–79.

HULTMARK, D., 1998. Quantification of antimicrobial activity, using the inhibition-zone assay. In: *Techniques in insect immunology* (eds. A. Wissner, G.B. Dunphy, V. J. Marmaras, I. Morishimma, M. Sugumaran and M. Yamakawa), USA Press.

HYRŠL, P., BÜYÜKGÜZEL, E. AND BÜYÜKGÜZEL, K., 2007. The effects of boric acid-induced oxidative stress on antioxidant enzymes and survivorship in *Galleria mellonella*. *Arch. Insect Biochem. Physiol*., **66**: 23–31.

IMAMURA, M., YANG, J. AND YAMAKAWA, M., 2002. cDNA cloning, characterization and gene expression of nitric oxide synthase from the silkworm, *Bombyx mori*. *Insect mol. Biol*., **11**: 257–265.

IMLAY, J.A., 2003. Pathways of oxidative damage. *Annu. Rev. Microbiol*., **57**: 395–418.

IMLAY, J. A. AND LINN, S., 1988. DNA damage and oxygen radical toxicity. *Science*, **240**: 1302–1309.

IMLER, J.L. AND BULET, P., 2005. Antimicrobial peptides in *Drosophila*: structures, activities and gene regulation. *Chem. Immunol. Allergy*, **86**: 1–21.

IRVING, P., UBEDA, J., DOUCET, D., TROXLER, L., LAGUEUX, M., ZACHARY, D., HOFFMANN, J., HETRU, C. AND MEISTER, M., 2005. New insights into *Drosophila* larval haemocyte functions through genome wide analysis. *Cell Microbiol*. **7**: 335–350.

JAIN, S.K., 1984. The accumulation of malonyldialdehyde, a product of fatty acid peroxidation, can disturb aminophospholipid organization in the membrane bilayer of human erythrocytes, *J. biol. Chem*., **25**: 3391–3394.

KANNAN, K. AND JAIN, S.K., 2000. Oxidative stress and apoptosis. *Pathophysiology*, **7**: 153–163.

KANOST, M.R. AND GORMAN, M., 2008. Phenoloxidases in insect immunity. In: *Insect immunity* (ed. N. E. Beckage), Academic Press, San Diego, pp. 69-96.

KAVANAGH, K. AND REEVES, E.P., 2004. Exploiting the potential of insects for *in vivo* pathogenicity testing of microbial pathogens. *FEMS Microbiol. Rev*., **28**: 101–112.

KIM, B,Y., LEE, K.S., CHOO, Y.M., KIM, I. JE, Y. H., WOO, S.D., LEE, S.M., PARK, H.C., SOHN, H. AND JIN, B.R., 2008. Insect transferrin functions as an antioxidant protein in a beetle larva. *Comp. Biochem. Physiol*., Part B, **150**: 161–169.

KORNBLUTH, S. AND WHITE, K., 2005. Apoptosis in Drosophila: neither fish nor fowl (nor man, nor worm). *J. Cell Sci*., **118**: 1779–1787.

KUMAR, S. AND DOUMANIS, J., 2000. The fly caspases. *Cell Death Differ*., **7**: 1039–1044.

KUMAR, S., CHRISTOPHIDES, G.K., CANTERA, R., CHARLES, B., HAN, Y.S., MEISTER, S., DIMOPOULOS, G., KAFATOS, F.C. AND BARILLAS-MURY, C., 2003. The role of reactive oxygen species on *Plasmodium melanotic* encapsulation in *Anopheles gambiae*. *Proc. natl. Acad. Sci. U.S.A*., **100**: 14139–14144.

LI, D., BLASEVICH, F., THEOPOLD, U. AND SCHMIDT, O., 2003. Possible function of two insect phospholipid-hydroperoxide glutathione peroxidases. *J. Insect Physiol*., **49**: 1–9.

MEISTER, A. AND ANDERSON, M.E., 1983. Glutathione. *Annu. Rev. Biochem*., **52**: 711-760.

MORTON, D., DUNPHY, G. AND CHADWICK, J., 1987. Reactions of haemocytes of immune and non-immune *Galleria mellonella* larvae to *Proteus mirabilis*. *Develop. Comp. Immunol*., **11**: 47–55.

NAPPI, A.J. AND VASS, E., 2001. Cytotoxic reactions associated with insect immunity. In: *Phylogenetic perspectives on the vertebrate immune system* (ed. E.L. Cooper), Kluwer Academic-Plemum. pp. 329–348,

NIMMO, D.D., HAM, P.J., WARD, R.D. AND MAINGON, R., 1997. The sand fly *Lutzomyia longipalpis* shows specific humoral responses to bacterial challenge. *Med. Veter. Ent*., **11**: 324–328.

OKHAWA, H., OHISHI, N. AND YAGI, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem*., **95**: 351-358.

PETERSON, T. M.L. AND LUCKHART, S., 2006. A mosquito 2-Cys peroxiredoxin protects against nitrosative and oxidative stresses associated with malaria parasite infection. *Free Radic. Biol. Med*., **40**: 1067–1082.

PETERSON, T. M. L., GROW, A.J. AND LUCKHART, S., 2007. Nitric oxide metabolites induced in *Anopheles stephensi* control malaria parasite infection. *Free Radic. Biol. Med*., **42**: 132–142.

POUPARDIN, R., R., REYNAUD, S., STRODE, C., RANSON, H., VONTAS, J. AND DAVID, J. 2008. Cross-induction of detoxification genes by environmental xenobiotics and insecticides in the mosquito *Aedes aegypti*: Impact on larval tolerance to chemical insecticides. *Insect Biochem. mol. Biol.*., **38**: 540–551.

PRAPANTHADARA, L., HEMINGWAY, J. AND KETTERMAN, A.J., 1993. Partial purification and characterization of glutathione S-transferases involved in DDT resistance from the mosquito *Anopheles gambiae*. *Pestic. Biochem. Physiol*., **47**: 119–133.

RADYUK, S.N., SOHAL, R.S. AND ORR, W.C., 2003. Thioredoxin peroxidases can foster cytoprotection or cell death in response to different stressors: over- and underexpression of thioredoxin peroxidase in *Drosophila* cells. *Biochem. J*., **371**: 743–752.

RAFF, M., 1998. Cell suicide for beginners. *Nature*, **396**: 119–122.

RICHARDSON, H. AND KUMAR, S., 2002. Death to flies: *Drosophila* as a model system to study programmed cell death. *J. Immunol. Meth.*, **265**: 21–38.

ROEKRING, S. AND SMITH D.R., 2010. Induction of apoptosis in densovirus infected *Aedes aegypti* mosquitoes. *J. Inverteb. Pathol.*, **104**: 239–241.

ROST-ROSZKOWSKAA, M.M., MACHIDAB, R. AND FUKUIB, M., 2010. The role of cell death in the midgut epithelium in *Filientomon takanawanum* (Protura). *Tissue and Cell*, **42**: 24–31.

RUGALE, C., DELBOSC, S., MIMRAN, A. AND JOVER, B., 2007. Simvastatin reverses target organ damage and oxidative stress in Angiotensin II hypertension: comparison with apocynin, tempol, and hydralazine. *J. Cardiovasc. Pharmacol*., **50**: 293–298.

STADTMAN, E.R., 1986. Oxidation of proteins by mixed-function oxidation systems: implication in protein turnover, aging and neutrophil function. *Trends biochem. Sci*., **11**: 11–12.

STOHS, S.J. AND BAGCHI, D., 1995. Oxidative mechanisms in the toxicity of metal ions. *Free Radic. Biol. Med*., **18**: 321–336.

SUGUMARAN, M., NELLAIAPPAN, K., AMARATUNGA, C. AND CARDINALE, S.T., 2000. Insect melanogenesis. III. Metabolon formation in the melanogenic pathway regulation of phenoloxidase activity by endogenous dopachrome isomerase (decarboxylating) from *Manduca sexta*. *Arch. Biochem. Biophys*., **378**: 393–403.

THOMAS, J., MAIORINO, M., URSINI, F. AND GIROTTI, A., 1990. Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. In situ reduction of phospholipid and cholesterol hydroperoxides. *J. biol. Chem*., **265**: 454–461.

TEODORO, J.G. AND BRANTON, P.E., 1997. Regulation of apoptosis by viral gene products. *J. Virol.*, **71**: 1739–1746.

TOWNSEND, D.M., 2007. S-glutathionylation: indicator of cell stress and regulator of the unfolded protein response. *Mol. Interv*., **7**: 313–324.

URSINI, F., MAIORINO, M., VALENTE, M., FERRI, L. AND GREGOLIN, C., 1982. Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxides. *Biochim. biophys. Acta*, **710**: 197–211.

URSINI, F., MAIORINO, M. AND GREGOLIN, C., 1985. The selenoenzyme phospholipids hydroperoxide glutathione peroxidase. *Biochim. biophys. Acta*, **839**: 62–70.

VALLES, S.M. AND PEREIRA, R.M., 2005. *Solenopsis invicta* transferrin: cDNA cloning, gene architecture, and up-regulation in response to *Beauveria bassiana* infection. *Gene*, **358**: 60–66.

VILMOS, P. AND KURUCZ, E., 1998. Insect immunity: evolutionary roots of the mammalian innate immune system. *Immunol. Lett*., **62**: 59–66.

WANG, Z., LU, A. LI, X., SHAO, Q., BEERNTSEN, B.T., LIU, C., MAD, Y., HUANG, Y., ZHU, H. AND LING, E., 2011. A systematic study on hemocyte identification and plasma prophenoloxidase from *Culex pipiens quinquefasciatus* at different developmental stages. *Exp. Parasitol.*, **127**: 135–141.

WEI, W., MA, B., LI, H.Y., JIA, Y., LV, K., WANG, G., ZHANG, J., ZHU, S., TANG, H., SHENG, Z. AND XIA, Z., 2010. Biphasic effects of selective inhibition of transforming growth factor beta1 activin receptor-like kinase on LPS-induced lung injury. *Shock*, **33**: 218–224.

YOSHIGA, T., HERNANDEZ, V. P., FALLON, A.M. AND LAW, J.H., 1997. Mosquito transferrin, an acute phase protein that is up-regulated upon infection. *Proc. natl. Acad. Sci. USA*, **94**: 12337–12342.

ZHAO, P., LU, Z., STRAND, M.R. AND JIANG, H., 2011. Antiviral, antiparasitic, and cytotoxic effects of 5,6-dihydroxyindole (DHI), a reactive compound generated by phenoloxidase during insect immune response. *Insect Biochem. mol. Biol*., **41**: 645-652.

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