Lipid Peroxidation and Oxidative Protein Products as Biomarkers of Oxidative Stress in the Autogenous Mosquito, *Aedes caspius*, Upon Infection with the Mosquitocidal Bacterium, *Bacillus thuringiensis kurstaki*

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Abstract.- It has recently been shown that *Bacillus thuringiensis kurstaki* (*Btk*) suppressed both immune and antioxidant responses in the autogenous mosquito, *Aedes caspius*. The purpose of the presented study herein was to investigate the concomitant oxidative stresses upon infection with *Btk*. Data showed elevation in the level of both lipid peroxidation and protein oxidation, as important oxidative stress biomarkers, in *Btk*-infected *Ae. caspius* mosquitoes. In larval mosquitoes, *Btk*-infection induced significant higher levels of both lipid peroxidation and protein oxidation at 12 and 24h post-infection compared to those of control ones. In adult mosquitoes, *Btk*-inoculation has induced significant higher lipid peroxidation at 12 and 24h post-inoculation compared to control ones, while *E. coli*-inoculation induced significant higher lipid peroxidation at 12h post-inoculation only compared to control ones. However, significant high protein oxidation was shown only at 12h post-*Btk* inoculation but not in *E. coli*-inoculated mosquitoes compared to control ones in each case. These data may indicate that oxidative stress was more pronounced in *Btk*-inoculated adult mosquitoes than that in *E. coli*-inoculated ones. Moreover, *Btk*-induced oxidative stress was more pronounced in larvae than that in adult mosquitoes. Consequently, these findings suggest that the oxidative stress-induced cellular damage in mosquito larvae may be considered as an important pathogenicity mechanism of *Btk* against host mosquito larvae.

**Keywords:** Lipid peroxidation, oxidative protein products, *Bacillus thuringiensis*, *Aedes caspius*, oxidative stress.

INTRODUCTION

Insects live in all environments everywhere on the globe. Thus they are exposed to different challenging factors such as pathogens and contaminants. Humoral defense against pathogens is one of the crucial immune responses that refer to antibacterial proteins and other immune-related molecules generated by the fat body and/or haemocytes (Kavanagh and Reeves, 2004). These molecules are released into haemolymph to immobilize and kill invading microorganisms or parasites (Vilmos and Kurucz, 1998; Morton et al., 1987) which involves the synthesis of a range of anti-microbial peptides (Boman, 1998; Imler and Bulet, 2005). Cellular immune responses refer to responses such as phagocytosis nodule formation and encapsulation which are directly carried out by haemocytes (Gillespie et al., 1997, Irving et al., 2005; Wang et al., 2011).

Although reactive oxygen species (ROS) are used by insect as cytotoxic materials against invading pathogens and parasites (Fang, 1999; Peterson and Luckhart, 2006), they also cause oxidative stresses in insect itself, leading to cellular damage (Fang, 1999). This is because 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

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**Abbreviations:** AOPP, advanced oxidative protein products; APS, *Aedes* physiological saline; *Btk*, *Bacillus thuringiensis kurstaki*; DENV-2, dengue virus; *E. coli*, *Escherichia coli*; 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; ROS, reactive oxygen species; SOD, superoxide dismutase; Prxs: peroxiredoxins.
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). Superoxide radicals generated by oxidative stress act as oxidants or reductants that lead to the production of hydroxyl radicals (Fridovich, 1995). Therefore, host protection against oxidative stress is vital for homeostasis and hence survival.

Antioxidant defense refers to producing particular antioxidants that keep the balance between oxidants and antioxidants levels. Insects express a suite of antioxidant enzymes that may form a concatenated response to an onslaught of dietary and endogenously produced oxidants leading to oxidative stress (Felton and Summer, 1995; Hyršl et al., 2007). These antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferases (GSTs) and ascorbate peroxidase (APOX) (Ahmad, 1995; Halliwell and Gutteridge, 1999; Radyuk et al., 2003). Transferrine and glutathione (GSH) are important antioxidants that help keeping this oxidative/antioxidative balance. The former, is an antioxidant that controls the level of free iron (Yoshiga et al., 1997) and has been shown to be upregulated 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. The later is a tripeptide thiol found in virtually all metazoans, mediates reaction catalyzed by GST (Meister and Anderson, 1983) as one of the important antioxidative mechanisms that allow insects to survive in a contaminated environment (Poupadina, 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 as 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 a previous study, 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 kurstaki (Ahmed, 2011). In this study, infection of mosquitoes with B. thuringiensis kurstaki (Btk) has been shown to induce suppression in antibacterial immune response in terms of low PO titer and antioxidant defense in terms of low GSH titer. Thus, it would be interesting to support these finding by investigating oxidative stress biomarkers in Btk-infected mosquitoes. Thus, in the study proposed herein, lipid peroxidation and protein oxidation will be investigated as important biomarkers of oxidative stress-induced pathogenicity of Btk-infection in the autogenous mosquito, Ae. caspius.

MATERIALS AND METHODS

Mosquito rearing

An autogenous Ae. caspius mosquitoes were reared under standard insectarium conditions (26°C, 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). After adult emergence, mosquitoes of the same age were used for the relevant experiments in this study. To maintain a stock of mosquito colony, they were kept accessing 10% glucose since blood meal is not urgently needed for triggering vitellogenesis (Clements, 1992).

Experimental bacteria

Mosquito-larvicidal bacterium, Bacillus thuringiensis var kurstaki (Btk) (serotype H-3a and 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, Btk bacteria (spores from the spore-cystal powders) or Escherichia coli (kindly provided by the Department of Food Sciences and Nutrition, Faculty of Food Sciences and Agriculture, King Saud University) were routinely incubated in nutrient broth (13 g/l) at 37ºC for 48 h in a rotary shaker at 200 rpm until a spectrophotometrical optical density OD698 of 0.5-0.7 is reached (Nimmo et al., 1997) using UV Visible Spectrometer (Ultrospec 2000, Pharmacia Biotech), then used for inoculating mosquitoes according to Nimmo et al. (1997) as briefed below.

**Infecting larvae with Btk**

A rearing tray of one liter distilled water containing 100 3rd instar larvae of Ae. caspius was left without treatment (control) or treated with LC50 (0.058mg/l) (Ahmed, 2011) of spore-cystal powders of Bacillus thuringiensis kurstaki (Btk) (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. Alive larvae were used for estimating lipid peroxidation or advanced oxidative protein products at 12 and 24h post-treatment as detailed below.

**Mosquito inoculation with bacteria**

Bacterial suspensions of Btk or E. coli (OD698 of 0.5-0.7) were prepared as detailed above. Fifty 6-days old adult mosquitoes 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 CaCl2) as trauma control, Btk or E. coli 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 x 16 x 16 cm each) under the usual standard rearing 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.

**Determination of lipid peroxidation (LP)**

Endogenous lipid peroxidation in mosquito homogenate was estimated spectrophotometrically following the method described by Okhawa et al. (1979), with some modifications, expressed in a nano-moles of malondialdehyde (MDA) per milliliter mosquito homogenate (nmole/ml). Fifty control or bacterial-infected 3rd instar larvae (with LC50 = 0.058mg/l), or fifty bacterial-inoculated 6-days old mosquitoes (with Btk, E. coli or APS) were homogenized in 500 ml of phosphate buffer (0.05 M, pH 7.2) containing 2mM ethylenediaminetetra-acetic acid (EDTA; Sigma), 0.5mM dithiothreitol (DTT; Fluka), 0.8mM phenylmethylsulphonyl fluoride (PMSF; Sigma) and 1.5% polyvinyl-pyrolidone (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). To the resulting mixture, 1 ml of 0.67% thiobarbituric acid (TBA) was added, shaken, and wormed for 30 min in a boiling water bath and 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 an UV Visible Spectrometer (Ultrospec 2000, Pharmacia Biotech).

**Determination of advanced oxidative protein products (AOPP)**

Protein oxidation was measured in homogenates (of larvae or adult mosquitoes) that prepared at 12 or 24h post-treatment by determining the AOPP levels spectrophotometrically (Rugale et al., 2007; Aiassa et al., 2007). One milliliter of 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 (Ultraspec 2000, Pharmacia Biotech) to calculate the chloramine-T equivalents protein products in µM/ml.

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 and AOPP 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 insect groups in each case (Morrison, 2002). Five replicates (five different mosquito groups in each case: N = 5) were carried out for better statistical analysis.

RESULTS
Measuring oxidative stress is not straightforward, and standardized methods are still lacking. The current study investigates the possibility that oxidants could induce stress that may result in damage to cellular lipid and protein molecules in Btk-infected mosquitoes. Thus, the current study focused mainly on lipid peroxidation and protein oxidation as important biomarkers of cellular collapse upon Btk infection in mosquito larvae and adults. This is based on modifying the characteristics of proteins and lipids which can be fractionated by size exclusion spectrophotometrically at 340 and 535 nm, respectively.

LP in larval mosquitoes
Third instar larvae of the autogenous Ae. caspius mosquito were exposed to LC₅₀ (0.058 mg/l) of B. thuringiensis (Btk) for 24h. Data from LP assay showed a significant higher MDA concentration in the homogenate of Btk-infected larvae compared to that of control ones at 12h post-treatment (3.667 ± 0.435 µmole/ml respectively) (P < 0.05, N = 5, student t-test) (Fig. 1). Moreover, MDA concentration was still significantly higher in Btk-infected larvae at 24h post-infection compared to that of control ones (2.92 ± 0.374 µmole/ml respectively) (P < 0.05, N = 5, student t-test) (Fig. 1).

LP in adult mosquitoes
Six-days old autogenous Ae. caspius mosquito were inoculated with B. thuringiensis (Btk), E. coli (Ec) or APS (control) prior to LP bioassay in terms of Malondialdehyde (MDA) titer. Larvae were treated with LC₅₀ (0.058 mg/l) of B. thuringiensis (Btk) spore-crystal powder or left without treatment (control). MDA titer was measured spectrophotometrically (at 535 nm) at 12 and 24h post-Btk infection. Error bars represent standard errors of means of 5 replicates (N = 5). Asterisk (*) represents significantly higher MDA level compared with control larvae (P < 0.05, two-sample t-test).

Fig. 1. Malondialdehyde (MDA) titer (µmole/ml) in third larval instars of the autogenous Ae. caspius mosquito. Larvae were treated with LC₅₀ (0.058 mg/l) of B. thuringiensis (Btk) spore-crystal powder or left without treatment (control). MDA titer was measured spectrophotometrically (at 535 nm) at 12 and 24h post-Btk infection. Error bars represent standard errors of means of 5 replicates (N = 5). Asterisk (*) represents significantly higher MDA level compared with control larvae (P < 0.05, two-sample t-test).

LP in adult mosquitoes
Six-days old autogenous Ae. caspius mosquito were inoculated with B. thuringiensis (Btk), E. coli (Ec) or APS (control) 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 Btk bacteria compared to that of control ones (18.33 ± 2.41 µmole/ml respectively) (P < 0.05, N = 5, student t-test) (Fig. 2). Moreover, MDA concentration was
significantly higher at 12h post-inoculation with E. coli compared to that of control ones (21.67 ± 2.56 n mole/ml, respectively) \( (P < 0.05, N = 5, \text{student t-test}) \) (Fig. 2). This may indicate that Btk and E. coli inoculation has significantly increased lipid peroxidation in adult autogenous Ae. caspius mosquitoes.

Concentration in E. coli-inoculated mosquitoes was similar to that of control ones at 24h post-inoculation (4.2 ± 0.94 n mole/ml, respectively) \( (P > 0.05, N = 5, \text{student t-test}) \) (Fig. 3). This may indicate that Btk inoculation has a long-term adverse oxidative effect on adult mosquitoes comparing to that of E. coli inoculation in terms of lipid peroxidation.

For investigating long-term oxidative stress upon inoculation with Btk or E. coli, MDA levels were measured at 24h post-inoculation with either of bacterial types in comparison with control mosquitoes. Data from LP assay showed significant higher MDA concentration in the homogenate of mosquitoes at 24h post-inoculation with Btk bacteria compared to that of control ones (7.267 ± 0.878 n mole/ml, respectively) \( (P < 0.05, N = 5, \text{student t-test}) \) (Fig. 3). However, MDA concentration in E. coli-inoculated mosquitoes was similar to that of control ones at 24h post-inoculation (4.2 ± 0.94 n mole/ml, respectively) \( (P > 0.05, N = 5, \text{student t-test}) \) (Fig. 3). This may indicate that Btk inoculation has a long-term adverse oxidative effect on adult mosquitoes comparing to that of E. coli inoculation in terms of lipid peroxidation.

**AOPP in larval mosquitoes**

The oxidative effect of Btk infection on proteins in the 3rd larval instar was spectrophotometrically investigated in larval homogenate. This experiment showed that infecting larvae with Btk induced a significant increase of AOPP compared to control ones (77.21±4.48 µmole/ml, respectively) \( (P < 0.05, N = 5, \text{student t-test}) \) (Fig. 4) at 12 h post-inoculation (Fig. 4). This significant increase in AOPP was also
monitored at 24h post-infection compared to that of control larvae (78.05±0.28 vs 48.052±4.48 µmole/ml respectively) (P<0.05, N = 5, student t-test) (Fig. 4).

**AOPP in adult mosquitoes**

Protein damage, in terms of advanced oxidative protein products (AOPP), was investigated spectrophotometrically in 6-days old autogenous *Ae. caspius* mosquitoes upon inoculation with *B. thuringiensis* (Btk), *E. coli* (Ec) or APS (control). Data from bacteria-inoculated mosquito homogenates showed significant higher AOPP concentration in Btk-inoculated mosquitoes at 12h post-inoculation compared to that of control ones (42.81 ± 1.77 vs 35.14 ± 0.27 µmole/ml respectively) (P < 0.05, N = 5, student t-test) (Fig. 5). However, AOPP concentration in *E. coli*-inoculated mosquitoes was similar to that in control ones at 12h post-inoculation (35.752 ± 0.35 vs 35.14 ± 0.27 µmole/ml respectively) (P > 0.05, N = 5, student t-test) (Fig. 5). This may indicate that *Btk*, but not *E. coli*, has induced protein damage in adult autogenous *Ae. caspius* mosquitoes as early as 12h post-inoculation.

For investigating long-term protein damage in adult mosquitoes upon inoculation with *Btk* or *E. coli*, AOPP level was measured at 24h post-inoculation with either of bacterial types in comparison with control mosquitoes. Data from each experiment showed no significant differences in AOPP concentration in the homogenate of mosquitoes at 24h post-inoculation with *Btk* or *E. coli* compared to APS-inoculated ones (31.462 ± 0.125 or 31.605 ± 0.186 vs 31.437 ± 0.123 µmole/ml respectively) (P > 0.05, N = 5, student t-test) (Fig. 6). This may indicate that *Btk* has short-term protein damage effect in adult mosquitoes (Fig. 5) and *E. coli* has no protein damage effect on adult mosquitoes (Figs. 5, 6).
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Fig. 6. Oxidation of proteins to AOPP in 6-days old autogenous Ae. caspius mosquitoes at 24h post-inoculation with B. thuringiensis (Btk), E. coli (Ec) or APS (control). In each case, AOPP concentration was measured spectrophotometrically (at 340 nm) at 24h post-inoculation. Error bars represent standard errors of means of 5 replicates (N = 5). No significant differences in AOPP levels in bacterial inoculated mosquitoes comparing to control ones were detected (P > 0.05, two-sample t-test).

DISCUSSION

Phospholipids make up the bulk of all internal and external cellular membranes in eukaryotic cell. Most cellular membrane proteins are embedded in or attached to membrane phospholipids. The structure and function of membrane proteins depend on the precise composition of its immediate phospholipid environment. Oxidative stress is known to be associated with damaged cell membranes and cell death (Klein and Ackerman, 2003). Under normal conditions, a dynamic equilibrium exists between the production of reactive oxygen species (ROS) and the antioxidant capacity of the cell (Stohs and Bagchi, 1995). ROS includes superoxide, hydroxyl, peroxyl and nitric oxide (NO) free radicals (Stohs and Bagchi, 1995). Superoxide (O$_2^-$) is an important source of free radicals (Fridovich, 1986). Hydrogen peroxide

Fig. 7. Speculative schematic diagram suggesting a potential mechanism of oxidative stress in Btk-infected Ae caspius larvae (modified from Chauhan and Chauhan, 2006). Part A in the diagram (shown in black dotted arrows and numbering) illustrates the normal balance between generation of Reactive Oxygen Species (ROS), that called free radicals (3) caused by endogenous/exogenous pro-oxidants and the defense mechanism against ROS by antioxidants (4). Increasing in ROS levels may be due: I) exogenous environmental pro-oxidant factors (1) such as heavy metals, pollutants, toxins and microbial infections, II) increased production of endogenous pro-oxidants (2) such as NO, xanthine oxidase, homocysteine, or both which result in high level of ORS. On the other hand, antioxidants within the cell (4) such as ceruloplasmin, transferrin, superoxide dismutase, catalase and reduced glutathione are neutralizing these extra ORS. This oxidant/antioxidant balance is essential for normal functioning of these cells. Part B in the diagram (shown in red solid arrows and numbering) illustrates suggested mode of actions of Btk infection (5) in mosquito larva. Btk-infection may suppress both the immune responses of infected larvae and antioxidant defense (6 and 7, respectively) (Ahmed, 2011). Subsequently, this increases the level of ROS which imposes cellular oxidative stress. Increased oxidative stress (8) may lead to cytotoxicity (9) in terms of lipid peroxidation, protein oxidation, DNA oxidation, mitochondrial dysfunction and impaired energy production. These cellular abnormalities trigger apoptosis and/or necrosis (10) which ultimately results in cellular collapse (11).
(H$_2$O$_2$) reacts with iron to produce the highly reactive hydroxyl radical (McCord and Day, 1978) which causes the most cytotoxic effects as well as initiation of lipid peroxidation (McCord and Day, 1978).

In the current study, lipid peroxidation and advanced oxidation protein products bioassays, as important biomarkers for oxidative stress, were investigated in both larval and adult stages of the autogenous mosquito, Ae. caspius. Bioassays were carried out in adult mosquitoes (6 days-old) or 3$^{rd}$ larval instars at 12 and 24h post-$Btk$ 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 shown that ROS cause lipid peroxidation, protein carbonylation, DNA oxidation and glutathione depletion, leading to oxidative damage and alterations in radical scavenging enzymes in insect tissues (Ahmad, 1995). The current study reported significant 2.2 and 1.9 folds increase in TBA-reactive Malonyldialdehyde at 12 and 24h respectively post-infection with $Btk$ in mosquito larvae as compared to un-infected controls. This may indicate $Btk$-induced high lipid peroxidation that may have lead to cytotoxicity in infected larvae (Jain, 1984). Functional studies in mammals showed that phospholipid hydroperoxide glutathione peroxidases (PHGPx) prevent lipid peroxidation and protect biomembranes against oxidative stress (Ursini et al., 1982). This is because PHGPx are antioxidant enzymes that can directly reduce peroxidized phospholipids and cholesterol within membranes (Ursini et al., 1985; Thomas et al., 1990). Similar function for PHGPx in insect has also been demonstrated (Li et al., 2003). Based on these studies, the observed significant increase of lipid peroxidation upon $Btk$ infection in the current study may be attributed to $Btk$ suppression/deactivation of the production of PHGPx in infected mosquito larvae. Furthermore, $Btk$ has been shown to have suppressive effect on both glutathione production (an important antioxidant) and antibacterial immune activity in $Btk$-infected larvae (Ahmed, 2011) which may constitute a further mechanism of this elevated oxidative stress. Taken all together, I would suggest that these effects might be considered as pathogenesis mechanisms of $Btk$ against mosquitoes larvae (Fig. 7).

Exposure of proteins to ROS gives both side-chain oxidation and backbone fragmentation (Davies and Dean, 1997). Ultimately, these processes can result in the loss of structural or enzymatic activity of the protein and, hence, biological perturbations (Davies and Dean, 1997). Cytological dysfunction is believed to be correlated with oxidative stress via modification of biological structures and formation of AOPP. These protein products might be regarded as a family of compounds containing abundantly dityrosines, which allow cross linking, disulfide bridges, and carbonyl groups (Capeille're-Blandin et al., 2004). This may indicate that AOPP act as markers of oxidative stress, may contribute to cytological dysfunction (Descamps-Latscha et al., 2005). Oxidation of amino acid residues such as tyrosine, leading to the formation of dityrosine, protein aggregation, cross-linking and fragmentation is an example of ROS-mediated protein damage in vitro (Dean et al., 1997). This may have happened in $Btk$-infected larvae of the current study as they showed significant 2 and 1.6 folds increase in AOPP at 12 and 24h post-infection respectively compared to control ones. This elevation in AOPP was more pronounced in larvae compared to that in adult mosquitoes which showed significant 1.2 folds increase and no significant difference at 12 and 24h post-$Btk$ inoculation 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 to hydroxylation of tyrosine and oxidation of o-diphenols like dopamine to form quinones using molecular oxygen (O$_2$) (Sugumaran et al., 2000). Further metabolic processing of quinones results in the formation of the cytotoxic melanin (Cerenius and Soderhall, 2004). The melanogenesis-related cytotoxicity of melanin 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). Thus, in the evolutionary point of view, I may suggest that mosquitoes adapted a mechanism that protects them from this immune-related oxidative stressor via the antioxidant GSH (Erden et al., 2002; and reviewed by Christensen et al., 2005). Thus, the low GSH titer in Btk-infected mosquitoes that observed by Ahmed (2011) explains the elevated level of AOPP recorded in Btk-infected mosquitoes in the current study.

From the point of view of immune defense of insects, ROS inducers are involved in immune responses against foreign invaders. Herrera-Ortiz et al. (2011) investigated the involvement of H2O2 and Nitric oxide NO in the activation of the anti-malarial immune response in malaria-refractory strain of An. gambiae (compared to a susceptible strain) post-infected blood meal (Kumar et al., 2003; Herrera-Ortiz et al., 2004). NO is considered as a main immune factor against malaria development (Peterson et al., 2007; Herrera-Ortiz et al., 2011) as it induces the production of antimicrobial peptide in mosquito gut. NO also induced the production of antimicrobial peptide in uninfected Drosophila larvae (Foley and O’Farell, 2003) and in Bombyx mori (Imamura et al., 2002). To my knowledge, the concomitant oxidative stress in these insects as a result of NO induction is yet to be investigated. Mosquitoes, on the other hand, protect themselves against this immune-induced oxidative stresses through antioxidant defensive mechanism for their survival (Townsend, 2007). Evidence for this is study of Chen et al. (2011) who recorded endoplasmic reticular stress in mosquito cell line upon infection with dengue virus (DENV-2). This virus-induced cytotoxicity was characterized as changed mitochondrial membrane potential and generated superoxide production. On the other hand, they recorded significant elevation of GST minimized the resulting cellular damage as antioxidative response. Further evidence from malaria parasite infection in anopheline mosquitoes is the study of Peterson and Luckhart (2006) who observed the induction of oxidative stresses upon malaria infection that damaged mosquito tissues. They reported cellular defenses in mosquito that

minimized the resulting cellular damage via peroxiredoxins (Prxs), enzymes known to detoxify ROS, as antioxidative response. Data of the current study shows clearly that Btk has induced oxidative stress in infected mosquitoes. This could be explained by the observed low level of glutathione in Btk-infected mosquito larvae (Ahmed, 2011), and subsequently, interrupted the antioxidant-oxidant balance in infected mosquitoes. Hence, this Btk-induced oxidative stress may be considered as a mechanism behind high pathogenicity of Btk against mosquitoes (Fig. 7). Collectively, data of the current study demonstrated that Btk infection induced oxidative stress in Ae. caspius producing higher levels of ROS and this oxidative imbalance playing an important role in the larvicidal effect of this bacteria as shown for other toxins (Wei et al., 2010; Beckon et al., 2008). The short-term protein damage effect of Btk infection in adult mosquitoes (Fig. 5) compared to no protein damage in E. coli infected adult mosquitoes (Figs. 5, 6) may indicate that this oxidative stress is pathogenicity-dependent.

In conclusion, increased oxidative stress in Btk-infected mosquitoes (in terms of high levels of lipid peroxidation and protein oxidation) may be due to a) increased production of endogenous or exogenous pro-oxidants such as NO, xanthine oxidase, homocysteine (Stohs and Bagchi, 1995a), b) deficiencies of antioxidants (ceruloplasmin, transferrin, SOD, GPx, catalase, reduced glutathione) or (c) both. This increased oxidative stress may have lead to cytotoxicity which ends up with cellular death (Kannan and Jain, 2000) (Fig. 7). Thus, the suggested scenario that may have happened in Btk-infected larvae is 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). This scenario may explain the mechanism behind the high mosquitocidal pathogenicity of Btk within 24h post-infection. Therefore, I would suggest that the cytopathological effects of Btk in larval gut epithelial cells (Al-Robal et al., 2011) may have happened via this suggested scenario. Finally, the question now is "would the inhibition of ROS (through oral feeding of antioxidants) reduce..."
cytotoxicity (pathogenicity) of entomopathogenic microorganisms against beneficial insects and/or activation of endogenous ROS via adding exogenous antioxidant-inhibitors to the formula of biocontrol agents be in favor of biocontrol measures?” Although trials of activating the antioxidant defense has been recently took place (Alquicer et al., 2009), this exciting question awaits to be tackled.

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REFERENCES


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