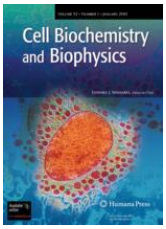

Mitochondrial P-Glycoprotein ATPase Contributes to Insecticide Resistance in the Cotton Bollworm, Helicoverpa armigera (Noctuidae: Lepidoptera)

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Mitochondrial P-glycoprotein ATPase contributes to insecticide resistance in the cotton bollworm, *Helicoverpa armigera* (Noctuidae: Lepidoptera)

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Abstract

Cotton bollworm, *Helicoverpa armigera*, is one of the most damaging polyphagous pests worldwide, which has developed high levels of resistance to commonly applied insecticides. Mitochondrial P-glycoprotein (Pgp) was detected in the insecticide-resistant strain of *H. armigera* using C219 antibodies, and its possible role was demonstrated in the efflux of xenobiotic compounds using spectrofluorometer. The TMR accumulated in mitochondria in the absence of ATP, and effluxed out in presence of ATP; the process of efflux was inhibited in the presence of ortho-vandate, an inhibitor of Pgp, in insecticide-resistant larvae of *H. armigera*. The mitochondria isolated from insecticide-resistant larvae were resistant to insecticide-induced inhibition of oxygen consumption and cytochrome c release. Membrane potential decreased in a dose-dependent manner in presence of higher concentration of insecticides (> 50 μM) in mitochondria of insecticide-resistant larvae. In conclusion, mitochondrial Pgp ATPase detected in the insecticide-resistant larvae influenced the efflux of xenobiotic compounds. Pgp might be involved in protecting the mitochondrial DNA and the components of the electron transport chain from damage due to insecticides, and contributing to the resistance to the deleterious effects of insecticides on the growth of insecticide-resistant *H. armigera* larvae.

Keywords *Helicoverpa armigera*, mitochondria, multidrug resistance, Pgp ATPase, insecticides.

Abbreviations FCCP, Carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone; MDR, Multidrug resistance; Pgp, P-glycoprotein; TMR, Tetramethylrosamine.

Introduction

Cotton bollworm/legume pod borer, *Helicoverpa armigera* (Hubner), is one of the most important constraints to crop production in Asia, Africa, Australia and the Mediterranean Europe. It is a polyphagous pest, and has been reported to attack more than 200 different species of plants [1]. Because of high population build up and severe crop losses, insecticides have been used on a large scale for controlling this pest. *H. armigera* has developed high levels of resistance to organophosphates, carbamates, synthetic pyrethroids and organochlorine insecticides [2, 3]. Insecticide resistance in *H. armigera* is due to the combined effects of insensitivity of acetylcholine esterase to insecticides, expression of higher levels of esterases, phosphatases and a specific protein called p-glycoprotein ATPase [3].

Insects exhibiting resistance to one group of insecticides at times also exhibit resistance to other classes of insecticides, referred as cross-resistance. This is similar to multidrug resistance (MDR) in cancer cells [4]. MDR in mammals is associated with over-expression of plasma membrane proteins that belong to the ATP-binding cassette (ABC) family [5]. The Pgp functions in ATP-driven efflux of drugs from the cells, and is presumed to be an important cause of failure of cancer chemotherapy [6]. Similar ABC proteins have been implicated in the resistance of many organisms to a vast and chemically diverse range of

toxic molecules, and this type of resistance has been observed throughout the course of evolution [7]. The role of Pgp-like proteins in insects merits study because such transporters may also contribute to insecticide resistance. A few studies have examined the possible role of Pgp-like proteins in the malpighian tubules of insects [8]. A Pgp homolog was identified as a nicotine pump in the blood-brain barrier of tobacco budworm, *Manduca sexta* L. [8], which results in a decreased level of nicotine in the brain, thereby protecting the tobacco hornworm from toxicity to the central nervous system. Organochlorine and organophosphorus pesticides have been reported to bind to Pgp, and exposure to such compounds increases MDR1 gene expression [4, 9]. Although Pgp is primarily located in the plasma membrane, it has also been detected in the nucleus [10], golgi apparatus [11] and in the mitochondria [12, 13].

Despite the similarities in metabolism between vertebrates and arthropods, insects specifically possess some features for requisite reactions, as a possible adaptive mechanism to environmental stress [14]. Successful management of insecticide resistance for agriculture pests depends not only on modifying the way insecticides are deployed, but also on understanding the molecular mechanisms responsible for development of resistance to insecticides. Insecticides are known to impair mitochondrial energy metabolism, generate oxidative stress and cause neuronal apoptosis in rats [15]. In view of the toxicity of the insecticides to the mitochondria and development of resistance to insecticides in *H. armigera*, the present study was undertaken to gain an understanding of the role of mitochondrial Pgp ATPase in the efflux of xenobiotic compounds and providing protection to the cell organelle in insecticide-resistant larvae of *H. armigera*.

Materials and Methods

Chemicals

Bovine serum albumin (BSA), ADP, ATP, and FCCP were purchased from Sigma Aldrich (Mumbai, India). Sucrose was purchased from Qualigens (Mumbai, India), tetramethylrosamine (TMR) was obtained from Molecular Probes (Eugene, OR, USA). Methylparathion (99.3%) and carbofuran (99%) were procured from Pesticide Analysis Laboratory, Gulbarga, India, while the other chemicals used in these studies were of analytical grade.

Insects

Insecticide-susceptible and -resistant strains of *H. armigera* larvae were obtained from the insect rearing laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. The larvae were reared on a chickpea based semi-synthetic diet under laboratory conditions at 27 ± 1 ˚C, 65 ± 5% RH, and 12 h photoperiod [16].

Bioassays of Insecticides

Methylparathion and carbofuran were incorporated into the artificial diet at different concentrations (0–100 µM). Third-instar larvae of insecticide-susceptible and -resistant strains were released into the insecticide containing diets separately. The initial weights of *H. armigera* larvae were measured before releasing in the artificial diets. There were three replications for each treatment in completely randomized design, and there were 10 larvae in each replication. The larval weights were recorded 5 days after initiating the experiment.

Isolation of Mitochondria

The fourth- and fifth-instar larvae were starved for 3 h, their midgut content removed, washed in cold distilled water, and then homogenized in Dounce homogenizer under cold conditions in isolation media [0.25 M sucrose solution containing 0.1% defatted BSA]. The homogenate was filtered through a moist muslin cloth, and the filtrate centrifuged at 800×g for 10 min at 4 ˚C. The residue was re-suspended in extraction buffer and centrifuged at 800 × g for 5 min. The supernatants from both the centrifugations were combined and centrifuged at 8,000×g for 10 min. The mitochondrial pellet was re-suspended in the reaction mixture, and used immediately for measuring oxygen consumption [17].

Mitochondrial Respiration

Polarographic determination of oxidative phosphorylation was made using oxygraph (Hansatech Instruments Limited, Bachofer, Reutlingen, Germany) fitted with a Clark-type oxygen electrode. The reaction system contained 5 mM HEPES buffer, pH 7.2, 50 mM sucrose, 120 mM KCl, 5.55 mM $MgCl_2$ and freshly isolated mitochondria in a total reaction volume of 1.5 ml. After the addition of the substrate (10 mM succinate), the rate of oxygen consumption was measured by the addition of 0.1 mM ADP [18]. Protein concentration was determined by Lowry's method using BSA as a standard [19].

Pgp ATPase Activity

ATPase activity was determined by quantitating the release of inorganic phosphate from ATP [20]. An aliquot of mitochondrial extract was incubated in 1 ml of ATPase assay medium (containing 2.5 mM ATP; 75 mM KCl; 5 mM $MgCl_2$; 0.5 mM EGTA; 2 mM ouabain; 3 mM sodium azide; 50mM Tris–HCl, pH 7.4) for 15 min at 37 °C. The reaction was terminated by the addition of 2 ml ice-cold stopping medium, consisting of 0.2% (w/v) ammonium molybdate, 0.9% SDS, 2.3% trichloroacetic acid, 1.3% (w/v) sulfuric acid, and freshly

prepared 1% (w/v) ascorbic acid. After 15 min incubation at room temperature, the released phosphate was quantitated colorimetrically at 660 nm.

Nucleotide Specificity

Nucleotide specificity was tested using AMP, ADP and ATP for the release of inorganic phosphate with the mitochondrial Pgp ATPase.

Electrophoresis

The mitochondrial fraction (200 mg/ml) was solubilized in 1% 3-[(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid (CHAPS) in 50 mM potassium phosphate (pH 7.2) and kept on ice for 10 min. The sample was spun at 12,000xg for 1 h to eliminate mitochondrial fragments. The supernatant was subjected to SDS-PAGE on 7.5% polyacrylamide gels according to Laemmli [21]. Glycoprotein staining was carried out with periodic acid-Schiff reagent using a procedure described by Gerard [22].

Western Blotting

The presence of a Pgp-like protein in the isolated mitochondria from the insecticide-resistant larvae was confirmed as described previously [23], using Western blotting with C219 antibodies, which were directed at the C-terminal NBD of mammalian Pgp.

Detection of Cytochrome c Release

Mitochondria (1 mg protein) isolated from insecticide-susceptible and -resistant larvae were incubated in 50 mM phosphate buffer, pH 7.2, containing 50 µM of insecticide

(methylparathion and carbofuran) for 1 h in the presence of 0.1 mM Ca^{2+}. The reaction mixtures were centrifuged at 12,000×g for 10 min at 4 ˚C to obtain the mitochondrial pellet. The supernatants were further spun at 12,000×g for 1 h to eliminate mitochondrial fragments. The supernatants were collected and the released cytochrome c was estimated according to Chamberlin [17].

Fluorometric Assay of Mitochondrial Pgp Transport Function

Mitochondrial fraction was prepared from insecticide-susceptible and -resistant *H. armigera* larvae as described above. The mitochondria were suspended in an isolation media on ice until the experiment was performed. Rhodamine, a known substrate for Pgp, was used as an indicator of Pgp activity [24], therefore, the rhodamine derivative, TMR was used to evaluate the transport function of mitochondrial Pgp in intact mitochondria in this study. All the fluorometric experiments were performed at room temperature using Cary Varian Eclipse fluorescence spectrophotometer in kinetic mode. Excitation was carried out at 550 nm (slit width, 5 nm) and the fluorescence emission was monitored continuously at 575 nm (slit width, 5 nm). The reaction mixture contained 50 mM sucrose, 0.02% BSA, 10 mM succinate, 200 nM TMR and 200 mg of mitochondrial protein. Movement of the dye was monitored in TMR pre-incubated mitochondria isolated from insecticide-susceptible and -resistant larvae by initiating the Pgp ATPase activity by the addition of 2.5 mM ATP, or by inhibiting Pgp using 1 mM sodium ortho-vanadate (an inhibitor of Pgp ATPase).

Mitochondrial Membrane Potential ($\Delta\psi$)

The transmembrane potential of the isolated mitochondria was evaluated according to the modified method of Braguini et al. [25], using rhodamine B, in the same reaction mixture used above for measuring the TMR transport. Fluorescence was measured in a Cary Varian

Eclipse fluorescence spectrophotometer in kinetic mode, with excitation and emission wavelengths of 550 and 575 nm, respectively, with a slit width of 5.0 for both excitation and emission. Mitochondria were energized with 5 mM sodium succinate, and 10 µM FCCP (carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone) was used as an uncoupler to diminish the membrane potential. Membrane potential was determined as $\Delta\psi = \Delta F/F_0$ x 100 (where, F_0 is the baseline fluorescence intensity (before the application of FCCP), and ΔF is the change in the fluorescence intensity between the baseline and after applying FCCP). The change in fluorescence intensity was used to compute the percentage change in TMR fluorescence intensity between the baseline level and the stimulus, which reflected the change in relative levels of $\Delta\psi$ [26].

Results

Bioassays of Insecticides on *H. armigera* Larvae

Both the insecticides inhibited the larval growth in a dose-dependent manner in insecticide-susceptible strain. At 100 µM, there were about 64% and 67% inhibition in larval weights in insecticide-susceptible larvae when fed on diets containing methylparathion and carbofuran, respectively, whereas, the insecticides were ineffective at lower concentrations (< 50 µM) in insecticide-resistant strain with an inhibition of about 36% and 30% larval weight when fed on diet containing methylparathion and carbofuran, respectively (Fig. 1).

Insecticides Inhibit Oxygen Consumption in Mitochondria

The untreated control mitochondria from *H. armigera* larvae consumed oxygen at the rate of 63.2 ± 5.2 nmoles/min/mg protein. At 100 µM concentration, methylparathion inhibited 77.8% and 39.4% of oxygen consumption in mitochondria isolated from insecticide-susceptible and resistant larvae respectively, when compared to untreated control mitochondria. At the same concentration, this insecticide inhibited 74.6% of oxygen

consumed in mitochondria isolated from insecticide-resistant larvae when the ATPase inhibitor sodium ortho-vanadate was included in the assay mixture, which is similar to the inhibition level observed in mitochondria isolated from insecticide-susceptible larvae (Fig. 2). Ortho-vanadate itself had no effect on ATP consumption by the mitochondria. Carbofuran produced a similar effect in insecticide-susceptible and -resistant larvae of *H. armigera*.

Insecticides Modulate Pgp ATPase Activity

Insecticides stimulated the ATPase activity of Pgp in isolated mitochondria at lower concentrations, but inhibited it at higher concentrations *in vitro*. At 40 μM concentration, methylparathion and carbofuran stimulated Pgp ATPase activity by 52% and 48%, respectively (Fig. 3). Substrate specificity tested in the direction of hydrolysis of various adenine nucleotides indicated that the enzyme was highly specific for ATP as substrate, and did not use ADP and AMP.

Pgp is Present in Insect Mitochondria

The presence of Pgp in *H. armigera* larvae mitochondria was detected by SDS-PAGE and confirmed by Western blotting using C219 antibodies, which are specific for the nucleotide binding domain of Pgp (Fig. 4). The molecular weight of mitochondrial Pgp was found to be 150 kDa as determined by SDS-PAGE.

Insecticide-Induced Cytochrome c Release from Mitochondria

Methylparathion and carbofuran induced the efflux of cytochrome c when incubated with isolated mitochondria from insecticide-susceptible larvae in the presence of Ca^{2+}. Cytochrome c levels in the supernatant were found to be 1.76 and 1.56 nmol/mg protein for methylparathion and carbofuran, respectively, whereas in the insecticide-resistant larvae, an insignificant amount of cytochrome c was detected (Fig. 5).

Functional Studies of TMR Transport by Mitochondrial Pgp

When TMR was incubated with mitochondria isolated from insecticide-susceptible larvae in the absence of ATP, the intensity of TMR decreased over time (Fig. 6(i)a), indicating the accumulation of TMR inside the mitochondria. TMR also accumulated in the mitochondria isolated from insecticide-resistant larvae in presence of ortho-vanadate, an inhibitor of Pgp, was included in the reaction mixture (Fig. 6(i)b). Addition of 2.5 mM ATP to the reaction mixture containing freshly isolated intact mitochondria from insecticide-resistant larvae pre-incubated with 200 nM TMR, resulted in a rapid increase in TMR fluorescence, which reached a steady-state level after 10 min (Fig. 6(ii)a). A reverse effect was observed with the mitochondria isolated from insecticide-susceptible larvae, where TMR fluorescence intensity decreased over time, though ATP was included in the assay buffer (Fig. 6(ii)b).

Effect of Insecticides on the Transport Function of Pgp ATPase in Intact Mitochondria

In the presence of insecticides, the efflux of TMR was reduced when the Pgp ATPase activity was initiated by adding ATP to the mitochondria isolated from insecticide-resistant larvae. This was observed for both the insecticides, methylparathion and carbofuran, and is shown for methylparathion (Fig. 7(i) b-d). The efflux of TMR declined in a dose-dependent manner (Fig. 7(ii)), with 30-40% inhibition at 50 µM. Both the insecticides inhibited the efflux of TMR, and competed effectively with TMR for transport via Pgp.

Effect of Insecticides on the Membrane Potential ($\Delta\psi$) in Intact Mitochondria

In mitochondria isolated from insecticide-resistant larvae, pre-incubation of insecticide at lower concentrations (< 50 µM) did not affect the membrane potential (data not shown), but progressively impaired the development of $\Delta\psi$ at higher concentrations (> 50 µM) in a dose-dependent manner (Fig. 8). The data have been presented in Table 1 for the mitochondria

isolated from insecticide-resistant and -susceptible larvae of *H. armigera* in the presence of methylparathion.

Discussion

Insecticide resistance in *H. armigera* is characterized by several features, one of which is over-expression of Pgp on the cell membrane [3]. Pgp is an ABC superfamily protein that exerts its function by pumping out drugs and other compounds from cells, and its expression in cancer cells is associated with MDR.

The presence of Pgp ATPase in mitochondria has been reported for several cancerous cell lines [12, 13]. In this study, we reported for the first time the presence of Pgp in the mitochondria of *H. armigera*, and demonstrated its transport function in intact mitochondria using fluorometric kinetic assay. The Pgp ATPase activity measured *in vitro* was enhanced in the presence of lower concentrations of insecticides but inhibited at higher concentrations. A similar biphasic pattern of stimulation and inhibition was previously reported for the interaction of insecticides with the mammalian Pgp [27]. The presence of Pgp in the mitochondrial fraction was confirmed using SDS-PAGE, and Western blotting using Pgp-specific C219 antibodies (Fig. 4). Similar results have been reported earlier involving cancerous cells lines [13]. The rate of oxygen consumption in mitochondria from insecticide-susceptible larvae decreased in the presence of insecticides in a dose-dependent manner, whereas, oxygen consumption in mitochondria from the insecticide-resistant larvae did not exhibit such inhibition at lower insecticide concentrations (< 60 µM) (Fig. 1). This observation might be due to the presence of Pgp, which could extrude the insecticide, and thus protect the respiratory enzyme complexes in the inner membrane of mitochondria from the toxic effect of insecticide. In the presence of ortho-vanadate, an inhibitor of Pgp, oxygen consumption by mitochondria from insecticide-resistant larvae was inhibited to a similar

level as those from insecticide-susceptible larvae. As such, ortho-vanadate had no effect on the rate of oxygen consumption, suggesting that Pgp is responsible for reduction of insecticide-induced inhibition of oxygen consumption in mitochondria from insecticide-resistant larvae.

Cytochrome c was released into the supernatant when insecticide was incubated with the mitochondria of insecticide-susceptible larvae, but there was no such effect in insecticide-resistant larvae. Thus, over-expression of Pgp in the mitochondria appears to protect insecticide-resistant larvae from the release of cytochrome c (Fig. 5). These results are in accordance with studies on mitochondria from multidrug-resistant cancerous cells, where the presence of Pgp exhibited a block in the release of cytochrome c from mitochondria into the cytosol [13]. The fluorescent probe TMR was previously used as a substrate for studying the transport function of reconstituted mammalian Pgp [28], and inhibition of TMR transport by several insecticides has been demonstrated [27]. Fluorometric kinetic assay was earlier used to evaluate the transport of rhodamine and its derivatives for Pgp reconstituted in membrane vesicles [23]. In the present study whole cell organelle was used to study the Pgp-mediated transport of TMR in spectrofluorometer in kinetic mode. TMR accumulated inside the mitochondria of insecticide-susceptible larvae in the absence of ATP, and a similar effect was observed with mitochondria isolated from the insecticide-resistant larvae when the inhibitor ortho-vanadate was added to the reaction mixture (Fig. 6(i)). In the presence of ATP, the dye was effluxed out of mitochondria (pre-incubated with TMR) from insecticide-resistant larvae, as evidenced by an increase in TMR fluorescence intensity (Fig. 6(ii)a). However, no increase in TMR fluorescence was observed in the presence of non-hydrolysable ATP analogs (data not shown). These results indicated that the process of TMR transport out of intact mitochondria required ATP hydrolysis by functionally active Pgp. Prior incubation of mitochondria isolated from insecticide-resistant larvae with insecticides showed that there

was a dose-dependent decrease in TMR efflux, indicating that the insecticides competed with TMR and inhibited the net transport of the dye (Fig. 7(i) and 7(ii)). These results suggested that Pgp present in the mitochondrial membrane is functionally active, is involved in the efflux of insecticides, and oriented in a direction which pumps the insecticides out to cytosol. The orientation of Pgp in the mitochondrial membrane is still debatable. Some of the reports have suggested that Pgp is oriented in mitochondria of MDR cells outside-in (i.e., with its NBDs facing the cytosol), which might lead to mitochondrial accumulation of drugs [12]. It has also been reported that it is oriented inside-out (i.e., with its NBDs facing the mitochondrial interior), where it would efflux drugs from the cell organelle into the cytosol [13]. However, these studies were carried out using flow cytometry, where accumulation of the dye was measured inside the cell organelle. In the present work, the movement of TMR was traced over time in intact mitochondria, using a spectrofluorometer. We observed accumulation of dye in both the insecticide-susceptible and -resistant larvae in the absence of ATP. However, in the presence of ATP, the active transport of the dye was recorded as an increase in dye fluorescence intensity, indicating the efflux of dye in mitochondria (pre-incubated with TMR) in insecticide-resistant larvae. This process was inhibited in presence of ortho-vanadate, an inhibitor of Pgp, suggesting that the orientation of Pgp is inside-out, so that it could extrude the insecticide molecules from the mitochondria. The resistance to inhibition of oxygen consumption by insecticides observed in mitochondria from insecticide-resistant larvae can thus be explained by the TMR efflux experiments. Ling et al. [29] observed that Pgp is over-expressed in the mitochondria of a human hepatoma cell line depleted of mitochondrial DNA, where it facilitates the efflux of chemotherapeutics into the cytosol.

In the presence of insecticides, the membrane potential progressively decreased in a dose-dependent manner in the mitochondria isolated from insecticide-susceptible larvae.

Similar results have been reported by Braguini et al. [25]. Membrane potential was not affected at lower concentrations of the insecticides (< 50 µM) in the mitochondria isolated from insecticide-resistant larvae (data not shown), but at higher concentrations (> 50 µM), $\Delta\psi$ decreased in a dose-dependent manner (Fig. 8). This behavior likely arises because Pgp ATPase activity is stimulated at lower insecticide concentrations, and hence the insecticide is pumped out without affecting the membrane potential. The mitochondrial membrane potential is relatively high in cancerous cells [30]; in contrast, $\Delta\psi$ was found to be similar in the mitochondria isolated from insecticide-susceptible and -resistant larvae (Table 1). Therefore, mitochondria of insecticide-resistant larvae possibly possess some unique characteristics of the cancerous cells such as over-expression of functionally active Pgp, but with normal mitochondrial characteristics such as generation of ATP, and normal mitochondrial $\Delta\psi$. This could be an adaptation in insects, providing protection to the cell organelle from insecticides. This type of adaptation may be acquired by insects as a result of prolonged exposure to insecticides. The reduced inhibition of larval growth in insecticide-resistant strain of *H. armigera* larvae at lower concentrations of insecticides (< 60 µM) may be attributed to the decrease in inhibition of oxygen consumption, active transport of insecticides when pre-incubated with mitochondria, inhibition in disruption of membrane potential and inhibition in the release of cytochrome c *in vitro*.

In conclusion, the present study demonstrated that Pgp is expressed in the mitochondrial membrane of insecticide-resistant *H. armigera* larvae. Pgp is functionally active and works like a pump to extrude insecticidal molecules from the mitochondria. In insecticide-resistant *H. armigera* larvae, this mechanism possibly involved in the protection of mitochondrial DNA and the components of the electron transport chain from damage due to insecticides.

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Figure legends:

Fig. 1. Bioassays of insecticides. Third-instar larvae of insecticide-susceptible and -resistant strains were fed on diet containing varying concentrations of methylparathion (white and dark gray color) and carbofuran (gray and black color), respectively. The data represents the Mean ± SD (n = 3) (differences between control and insecticides-fed larvae were analyzed using analysis of variance (ANOVA). F-test was used to judge the significance of differences between the treatments, while the least significant difference (LSD) was used to test significance of differences between the means for the treatments and the untreated control at *$p < 0.05$).

Fig. 2. Effect of insecticides on mitochondrial respiration when succinate was used as oxidizable substrate. Insecticide was incubated with the mitochondria for 2 min prior to addition of succinate. Rate of oxygen consumed was measured in mitochondria isolated from insecticide-susceptible larvae in presence of methylparathion (a) and carbofuran (b), and mitochondria isolated from insecticide-resistant larvae in the presence of methylparathion and carbofuran in the assay buffer containing ortho-vanadate (c and d, respectively), and without ortho-vanadate (e and f, respectively). Data points represent the Mean ± SD (n = 3).

Fig. 3. Effect of methylparathion and carbofuran on mitochondrial Pgp ATPase activity. Pgp ATPase activity was assayed by incubating different concentrations of methylparathion and carbofuran for 2 min prior to the addition of ATP. The graph shows percentage activity as compared to the control (100%, in the absence of insecticides). Both the insecticides, methylparathion (♦), and carbofuran (■) stimulated ATPase activity at lower concentrations and inhibited it at higher concentrations. Data points represent the Mean ± SD (n = 3).

Fig. 4. SDS-PAGE profile and Western blot analysis for detection of mitochondrial Pgp ATPase expression in *H. armigera.* (a) Proteins from mitochondrial lysate were resolved by

SDS-PAGE (7.5%) and the gel was stained with Coomassie blue, with lane 1 containing molecular weight markers (29 – 205 kDa); lane 2, CHAPS-dissolved mitochondrial extract (100 µg); lane 3, ammonium sulfate precipitated proteins from mitochondrial extract; lane 4, glycosylation of Ha-Pgp. (b) Detection of Pgp in the mitochondrial fraction of insecticide-susceptible and -resistant larvae by Western blotting using C219 antibodies.

Fig. 5. Detection of cytochrome c release. Cytochrome c release was estimated by incubating mitochondria (1 mg protein) isolated from insecticide-susceptible and -resistant larvae in presence of methylparathion (**a**) and carbofuran (**b**). The data represents Mean ± SD (n = 3). Differences between control and incubations containing insecticides were analyzed using analysis of variance (ANOVA). F-test was used to judge the significance of differences between the treatments, while the least significant difference (LSD) was used to test significance of differences between the means for the treatments and the untreated control at $*p < 0.05$).

Fig. 6(i). Pgp-mediated transport of TMR in intact mitochondria. The intensity of TMR fluorescence is reported in arbitrary units. Accumulation of TMR in intact mitochondria isolated from (a) insecticide-susceptible larvae ($\Delta F = 1.35 \times 10^{-2}$ a.u. min^{-1} mg^{-1} protein); and (**b**) insecticide-resistant larvae in the presence of ortho-vanadate ($\Delta F = 1.58 \times 10^{-2}$ a.u. min^{-1} mg^{-1} protein). ΔF is directly proportional to the amount of TMR accumulated in the intact mitochondria (it is the difference between the initial intensity of TMR and the intensity at the end of the reaction in the medium). (ii) Transport of TMR in mitochondria isolated from insecticide-resistant larvae (a) and insecticide-susceptible larvae (b), when ATP was added in the assay buffer as indicated in the trace. The traces are the representative of three biological samples.

Fig. 7(i). Effect of insecticides on the Pgp-mediated transport of TMR in intact mitochondria isolated from insecticide-resistant larvae. Mitochondria were pre-incubated with TMR and the insecticide for 2 min in assay buffer and energized with 5 mM sodium succinate. Pgp ATPase activity was initiated by the addition of ATP as indicated in the traces in control mitochondria (a), and incubations containing methylparathion at 10 µM (b), 30 µM (c) and 50 µM (d) concentrations. (ii) Inhibition of the Pgp-mediated transport of TMR by methylparathion (▲) and carbofuran (■) in intact mitochondria isolated from insecticide-resistant larvae. The percentage efflux of the TMR intensity was calculated from Fig. 7(i), as a change in the fluorescence intensity between the baseline (F_0) and the steady-state reached after applying ATP (F), relative to the control mitochondria. The traces are the representative of three biological samples.

Fig. 8. Effect of methylparathion on membrane potential. The membrane potential ($\Delta\psi$) of mitochondrial preparations (200 mg ml^{-1}) isolated from insecticide-resistant larvae was estimated by fluorescence changes of TMR (200 nM) in a medium containing 50 mM sucrose, 0.02% BSA, and energized with 5 mM sodium succinate. Insecticide was pre-incubated with mitochondria before adding succinate to the reaction mixture. FCCP was

added as indicated to collapse Δψ. The traces are the representative of three independent experiments: (**a**) control, and in presence of methylparathion at 50 µM (**b**), 100 µM (**c**) and 150 µM (**d**) concentrations.

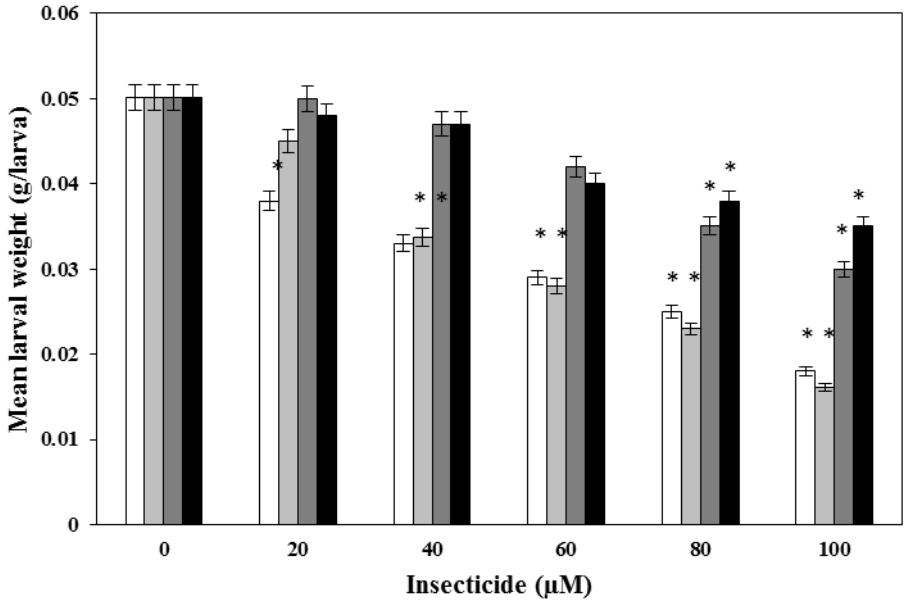

Figure 1

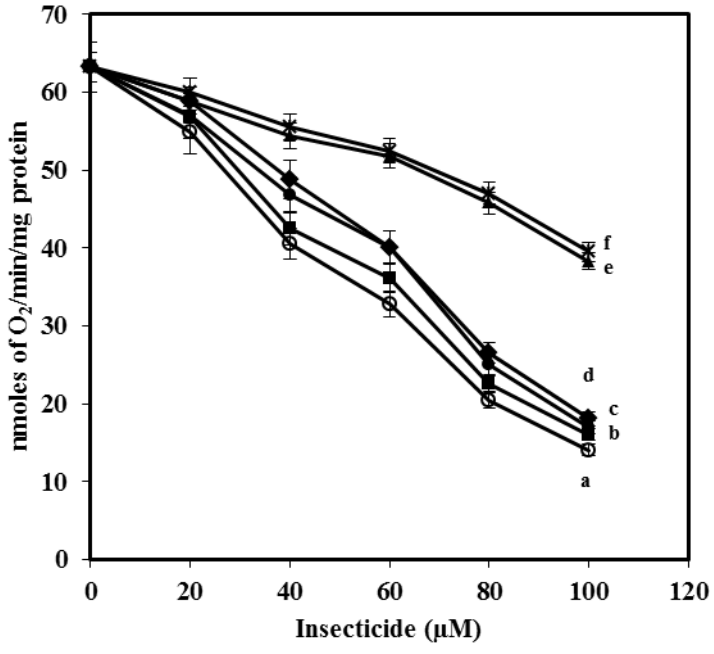

Figure 2

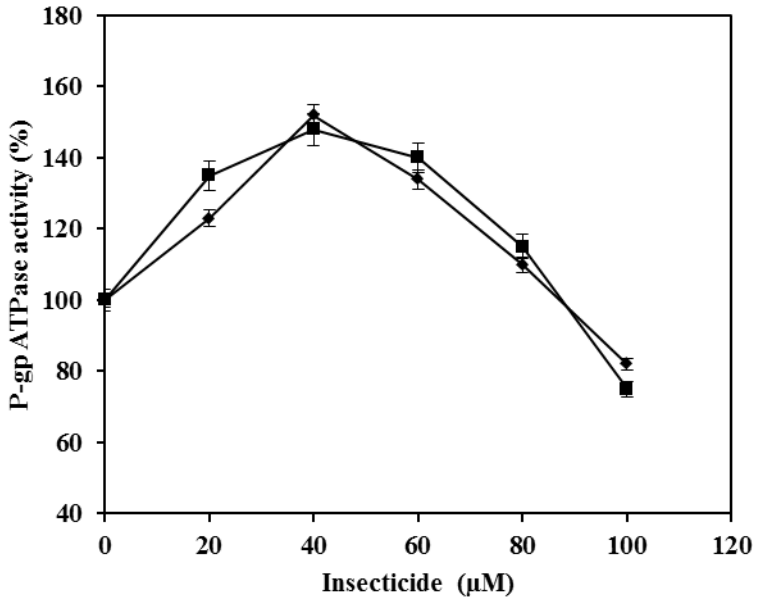

Figure 3

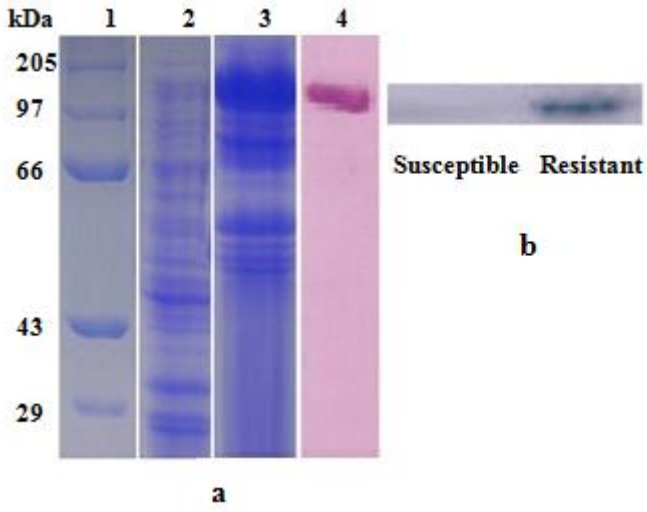

Figure 4

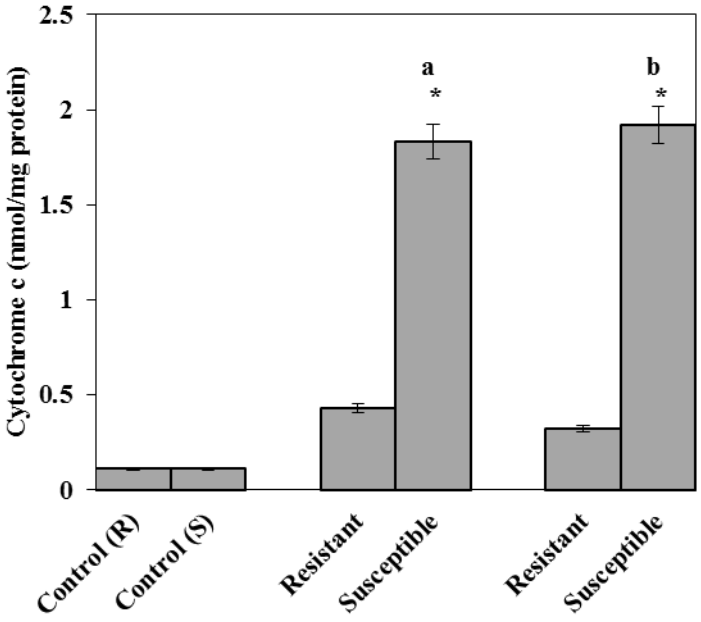

Figure 5

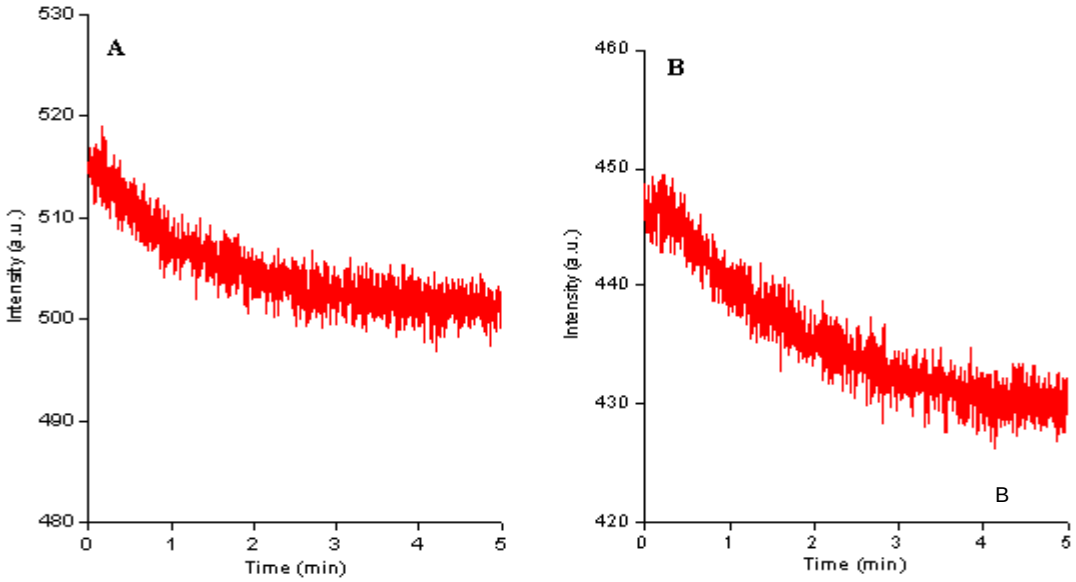

Figure 6(i)

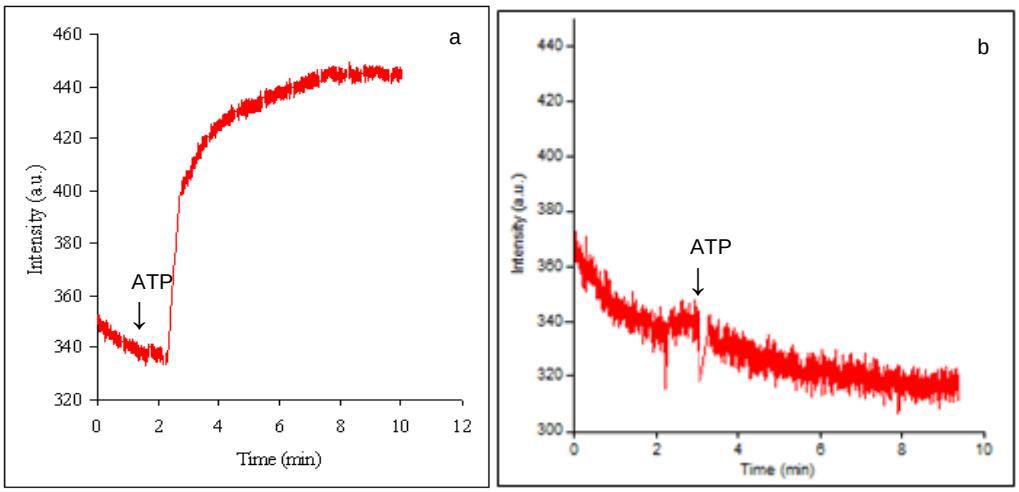

Figure 6(ii)

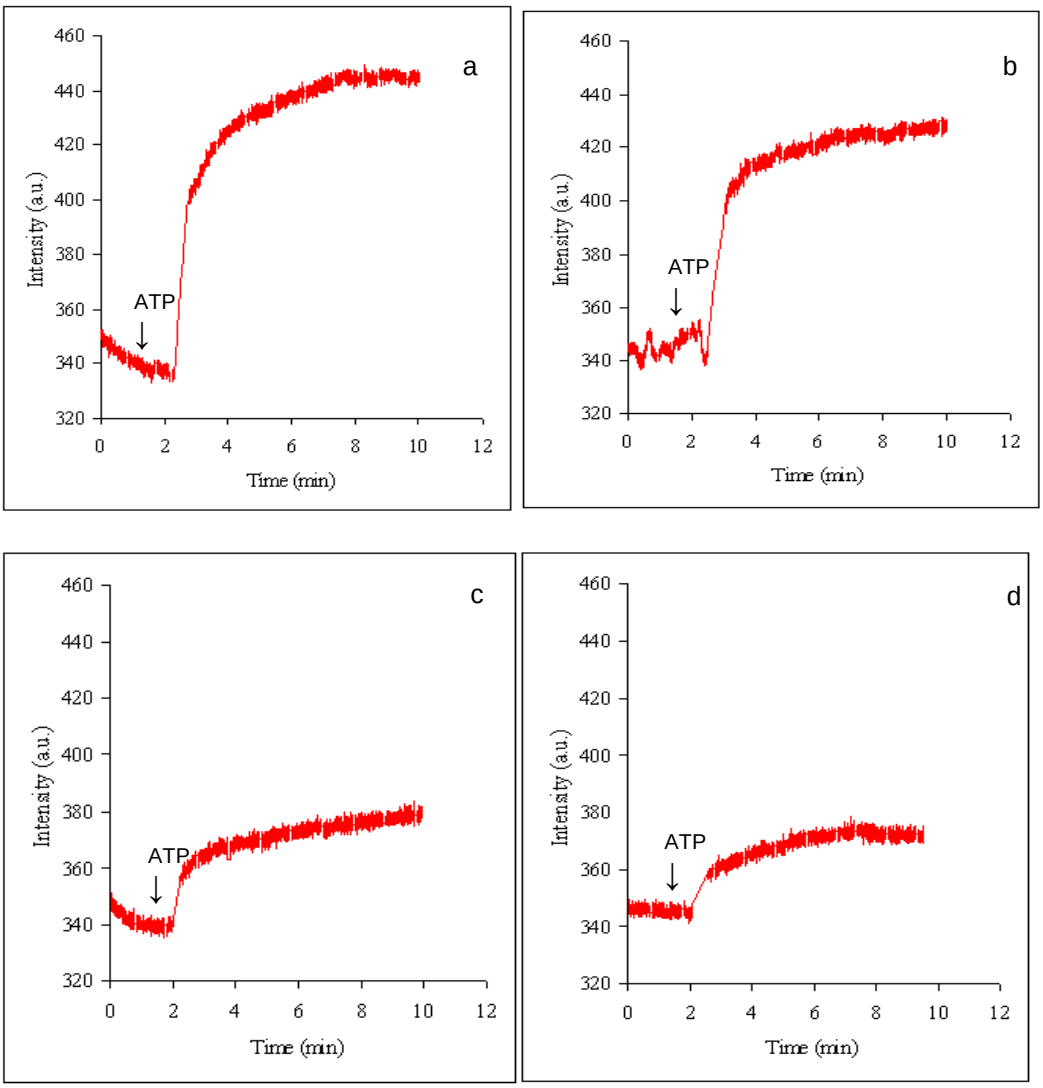
a
b
c
d
ATP
Intensity (a.u.)
Time (min)
460
440
420
400
380
360
340
320
0
2
4
6
8
10
12

Figure 7(i)

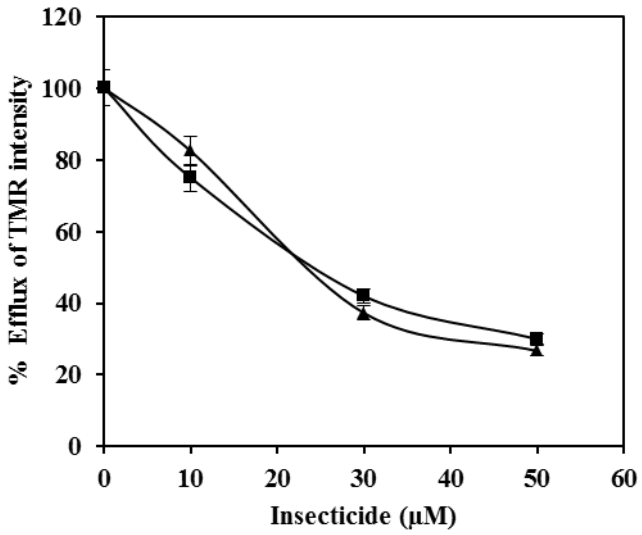
% Efflux of TMR intensity
Insecticide (µM)
120
100
80
60
40
20
0
0
10
20
30
40
50
60

Figure 7(ii)

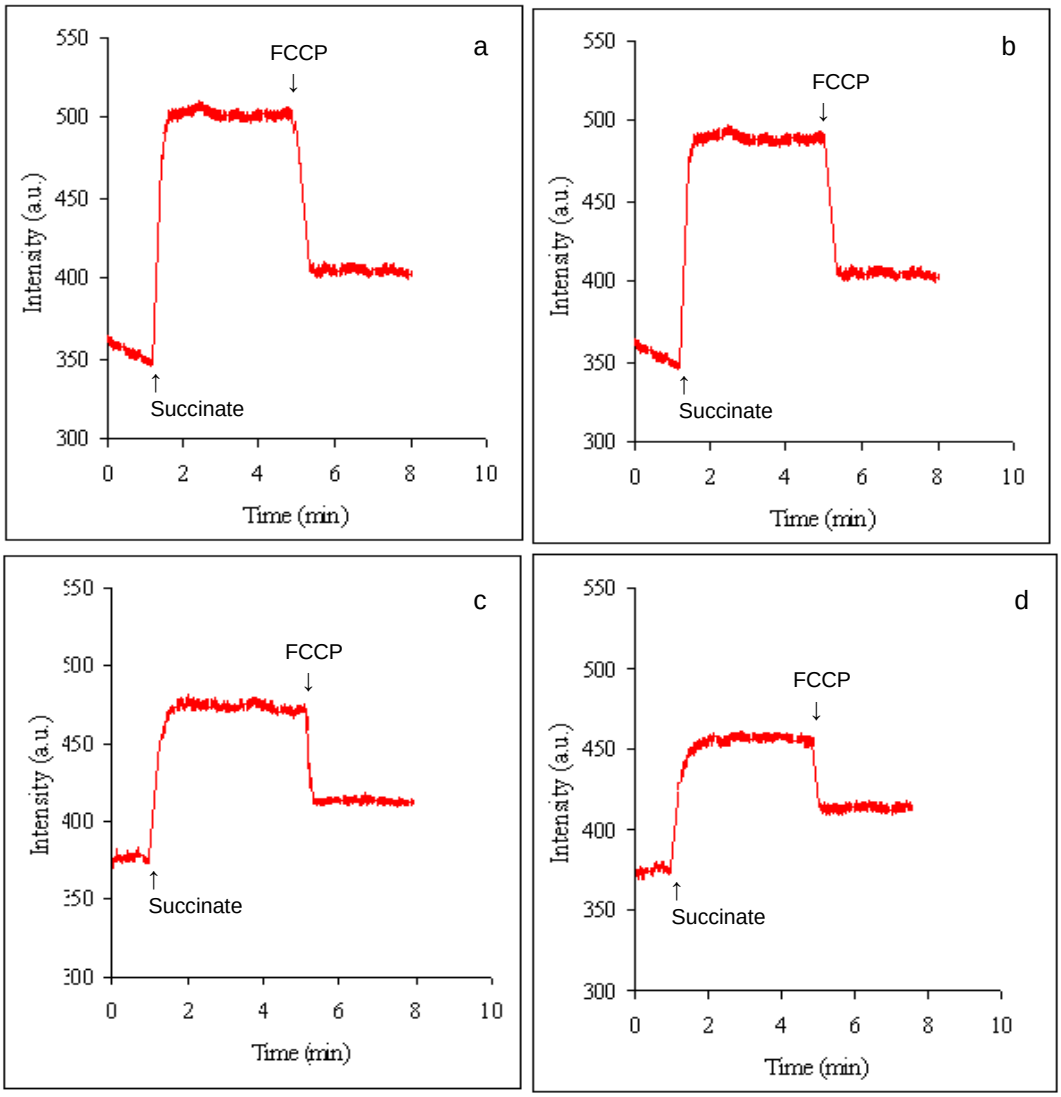

Figure 8

Table 1: Effect of methylparathion on the membrane potential in mitochondria isolated from

Insecticide	Δψin mitochondria isolated from insecticide-resistant larvae (%)	Δψin mitochondria isolated from insecticide-susceptible larvae (%)
Control	25 ± 0.24	25 ± 0.24
50 µM	20 ± 0.12*	15.46 ± 0.04*
100 µM	18.75 ± 0.15*	12.98 ± 0.08*
150 µM	12.5 ± 0.07*	8.48 ± 0.03*

insecticide-resistant and -susceptible larvae. The data represents the Mean ± S.D. (n = 3).

(Differences between control and incubations containing insecticides were analyzed using analysis of variance (ANOVA), F-test was used to judge the differences obtained by the ANOVA. *Significantly different from control, $p < 0.05$).