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Effect of *Staphylococcus aureus* Infection on Biochemical and Antioxidant Activities of Fifth Instar Silkworm Larvae (*Bombyx mori L.*)

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Abstract

In the present study silkworm used as a model animal for evaluating the pathogenicity of *Staphylococcus aureus*. The fifth instar silkworm larvae was used and infected by intrahaemocoelic injection of bacterial sample. The haemolymph was collected from the infected and control group larvae at 6, 12, 18 and 24 hours of post infection and stored at -4°C in eppendorf tubes to use. Lipid peroxidation, phenol oxidase and acid phosphatase activity were estimated in the haemolymph of control and infected group. This indicated that there was a gradual increase in lipid peroxidation, phenol oxidase and acid phosphatase activities in infected group when compared with control group. The antioxidant enzyme activities were estimated in the haemolymph of control and infected group. We found that the antioxidant enzyme activities were decreased after 24 hours of infection with *S. aureus*. The silk glands were removed and the wet weight was measured, the wet weight of the silk glands was decreased on the 24 hours of infection when compared with control group.

Keywords: Acid phosphatase; Haemolymph; Lipid peroxidation; Phenol oxidase; Silk gland

Introduction

Silkworm is a domesticated and economically important insect, being a primary producer of silk. The silkworm larval infection model has been proposed as a system in which to evaluate novel antimicrobial agents [1]. Microorganisms that are pathogenic to humans, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*, have lethal effects in silkworms when injected into the haemolymph [2-5]. There are several advantages to using silkworms as an infection model, such as low cost, the absence of ethical problems that are associate with the use of mammals and a body size large enough to handle while injecting sample solution into the haemolymph [3].

Staphylococcus is a genus of Gram positive, non-spore forming cocci found in the human respiratory tract and on the skin. *S. aureus* can cause serious infections such as skin, bloodstream infections (Bacteremia), pneumonia, bone and joint infections. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of hospital acquired infections that are becoming increasingly difficult to combat because of emerging resistance to all current antibiotic classes [6]. These strains appear to contain particular factors or genetic backgrounds that enhance their virulence [7]. *S. aureus* produces a wide variety of exotoxins that contribute to its ability to colonize and cause disease in mammalian hosts. This includes four hemolysins (alpha, beta, gamma, and delta), nucleases, proteases, lipases, hyaluronidase and collagenase. The main function of these exotoxins to convert local host tissues into nutrients required for bacterial growth [8]. Alpha-toxin, the major cytotoxic agent elaborated by *S. aureus*, was the first bacterial exotoxin to be identified as a pore former [9]. Toxic shock syndrome toxin (TSST) is the super antigen of the *S. aureus*, it causes toxic shock syndrome (TSS) by stimulating release of IL-1, IL-2, TNF-α and other cytokines [10].

S. aureus infection caused a marked decrease in plasma antioxidant defence systems illustrated by significant reductions in plasma paraoxonase activity, thiol concentrations in infected rabbits [11]. *S. aureus* infection causes decrease in superoxide dismutase activity, glutathione peroxidase activity, catalase activity and accumulation of lipid peroxidative products (MDA) in infected animal models by reduction in plasma paraoxonase activity, thiol concentrations. Formation of reactive oxygen species (ROS) and lipid peroxidation is a common phenomenon during infectious diseases. These free radicals causes' tissue injury and cell death, lipids of cells membranes and organelles are frequently damaged, resulting in lipid peroxidation. Staphylococcal infections are typically associated with death of tissue and evidence suggests intracellular bacteria are capable of inducing apoptosis [12]. *S. aureus* induce apoptosis has been reported in epithelial cells [13-15], keratinocytes [16], endothelial cells [17] and osteoblasts [18].

Materials and Methods

Rearing of *Bombyx mori* larvae

Fifth instar day sixth larvae of CSR-4 strain was reared in the laboratory by the improved method of rearing technique [19]. The silkworms are maintained on mulberry leaves at a temperature of 27°C and relative humidity of 75%. The life span of the silkworm under these conditions was 30-32 days.

Preparation of bacterial sample

Mannitol salt agar (MSA) medium was prepared a loopful sample of *S. aureus* bacterial sample was streaked on MSA and incubated at 37°C

for 24 hours. After incubation *S. aureus* formed golden yellow color colonies on MSA medium.

Inoculation of bacteria in Luria broth (LB)

A loopful of bacteria was taken with the help of a loop and streaked onto Luria broth (LB 1000 ml) and incubated at 37°C overnight. The inoculated LB sample was centrifuged for 15 min at 4000 rpm. By discarding the supernatant, pellet sediment at the bottom of the tube was dissolved in 100 ml of distilled water. The number of bacteria cells in the bacterial culture suspension was calculated by colony-forming unit (CFU).

Infection of silkworm larvae with bacterial strain

Fifth instar silkworm larvae were divided in to two groups, each group consisting of 20-30 larvae. One group was infected with the bacterium (3×10^5 cfu/ml to 1×10^8 cfu/ml) by intrahaemocoelic injection of bacterial sample. A similar number of larvae were injected with distilled water and considered as a control. Both control and infected larvae were reared under room temperature. The time of infection was recorded and the haemolymph was collected from the infected and control group larvae at 6, 12, 18 and 24 hours, post infection and stored at -4°C in eppendorf tubes to use.

Lipid peroxidation assay

To 0.5 ml of the homogenate (haemolymph), 0.5 ml of buffer, 2 ml of TCA (Trichloroacetic acid) were added followed by 4 ml of TBA (Thiobarbituric acid). The contents were heated in a water-bath at 100°C for 20 min. After cooling and centrifugation, the absorbance of the supernatant was read at 535 nm. A reagent blank was prepared using water instead of homogenate. The extent of lipid peroxidation was expressed as n mole of MDA formed/mg protein using a molar extinction coefficient for MDA of 1.56×10^5 M^{-1} cm.

Phenol oxidase assay

The phenoloxidase activity was determined by the method of Horowitz and Shen [20]. The reaction mixture consisted of 1 ml of 0.02 M, 4-di hydroxyphenylalanine (DOPA), 3.9 ml of 0.1 M phosphate buffer, pH 6.0 and 0.1 ml of enzyme solution. After incubation at 30°C for 5 minutes the color intensity of dopachrome was measured at 490 nm.

Acid phosphatase assay

Acid phosphatase activities were determined by the liberation of p-nitrophenol from the appropriate p-nitrophenyl substrate according to the method reported by De Couet and Blest [21]. The assay mixture contained 10 µl of tissue homogenate and 200 µl of substrate solution (2 mg/ml p-nitrophenyl phosphate, 1 mg/ml bovine serum albumin in homogenization buffer with 0.1 mol/l TRIS maleate at pH 5.2). The enzyme reaction was carried out at 21°C and samples arrested at 15 min intervals by addition of 50 µl 4N NaOH over 1 hour period. The reaction mixtures were then centrifuged at 1000 g for 10 min at room temperature and remove precipitate. Blanks were prepared by addition of NaOH prior to enzyme solution. Color development was determined at 405 nm using spectrophotometer. Readings were compared to a standard curve prepared with p-nitrophenol, the activity of enzyme was represented by µmol/min/mg.

Estimation of protein in the haemolymph

The total protein content was determined with Folin Ciocalteau's reagent according to the Lowry et al. [22]. Aliquot of test sample was made up to 1.0 ml with distilled water and 5.0 ml of alkaline solution was added, mixed thoroughly and allowed to stand at room temperature for 10 min. Then 0.5 ml of Folin-Ciocalteau's reagent was added rapidly with immediate mixing and the intensity of the colour developed was read at 750 nm after 30 min. Values are calculated from the standard graph.

Glutathione peroxidase (GPx) assay

The assay mixture consisted of 0.02 ml enzyme source, 1.78 ml buffer, 0.06 ml GSH, 0.02 glutathione reductase and 0.06 ml NADPH. The reaction was initiated by the addition of 0.06 ml of H_2O_2. The change in absorbance was recorded at 1 min intervals at 340 nm and the specific activity was calculated using extinction co-efficient of 6.22 cm^{-2}/µmol for NADPH. The activity was expressed as n mole NADPH oxidized/min/mg protein.

Catalase assay

Catalase catalyzes the breakdown of H_2O_2 to H_20 and 0_2 the rate of decomposition of H_2O_2 measured spectrophotometrically at 240 nm as outlined by Beers and Sizer [23]. The assay system contained 1.98 ml of Phosphate buffer, 0.02 ml of the enzyme source and 1.0 ml of H_2O_2. The change in absorbance was monitored at 240 nm and specific activity was calculated using a molar absorbance index for H_2O_2 of 43.6. The specific activity was expressed as moles of H_2O_2 decomposed/min/mg protein.

Superoxide dismutase (SOD) assay

Superoxide dismutase activity was measured based on the ability of the enzyme to inhibit the autooxidation of pyrogallol according to the method of Marklund and Marklund [24]. The assay system contained 2.1 ml of phosphate buffer, 0.02 of enzyme source and 0.86 ml of distilled H_2O. The reaction was initiated with the addition of 0.02 ml of pyrogallol and change in absorbance was monitored at 420 nm. The percent inhibition was calculated on the basis of blank assay system. One unit of SOD was defined as the amount of enzyme required to inhibit the autooxidation of pyrogallol. The specific activity was expressed as unit/min/mg protein.

Measurement of silk gland weight

After anesthesia, the silkworms were placed on a cork board, and the head and tail were pinned to it. A small cut was made in the tail, from which the abdomen was opened longitudinally by running ophthalmological scissors toward the head, and the silk gland, midgut, located in the middle of the abdomen, were removed. The wet weight of the silk gland was measured using a balance just after removal [25].

Statistical analysis

All the results are expressed as means ± SE. Statistical analysis of the data was performed by Student's t-test and P value <0.05 was considered statistically significant.

Result and Discussion

Lipid peroxidation (LPO) assay

The malondialdehyde (MDA) concentration was increased in the haemolymph of silkworm larvae infected with *S. aureus* when compared with control group. The highest MDA concentration (4.65 MDA nmol/ml) was reported at 24 hours post infection with bacteria (Figure 1). The MDA concentration was the same as that of the control larvae at 6 hours of infection, later the MDA concentration gradually increased at 12, 18 and 24 hours post infection with bacteria. The results obtained testify that the high level of lipid peroxidation at 24 hours post inoculation may be the result of cells destruction by exo and endotoxins of *S. aureus*. Generally the ROS are formed in small quantities during the metabolism, however, the production of ROS is increased during the infection [26]. The formation of lytic pores in the plasmatic membrane and the disconnection of oxidative phosphorylation processes may result in the overproduction of reactive oxygen species (ROS) causes lipid peroxidation [27].

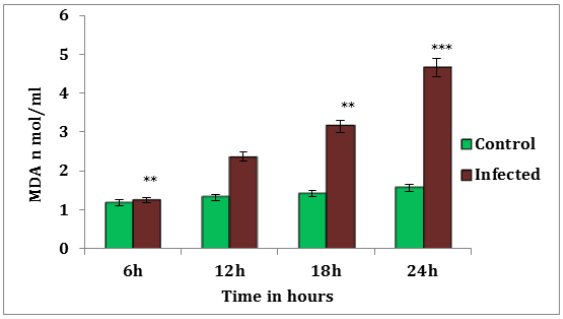

Figure 1: MDA concentration in the haemolymph of control and infected larvae of *B. mori* Values are mean ± SE from 8 silkworms in each group. Superscripts are significantly different at *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

Phenoloxidase (PO) assay

The phenol oxidase (PO) activity increased in the infected larvae when compared with control group at 12, 18 and 24 hours post infection (Figure 2) which indicates that PO activity was highly active against *S. aureus*. In melanization cascade, PO is the terminal enzyme, play an important role in defense mechanisms in invertebrates [28]. Phenol oxidase that catalyses the hydroxylation of tyrosine to DOPA (dihydroxylphenyl alanine) and the oxidation of DOPA to dopaquinone, is usually found as pro enzyme in the haemolymph and activated in response to defense reactions [29]. PO is present throughout the body of insects including the open circulatory system of haemolymph and haemocytes activated by the microbial factors. Activated prophenol oxidase (proPO) plays an important role in cuticular melanization of foreign organisms and sclerotization.

Acid phosphatase assay

The acid phosphatase activity was increased in infected larvae from 18 hours and 24 hours of post infection when compared with control group (Figure 3). The major changes were not observed in the acid phosphatase during 6 hours and 12 hours post infection when compared with control group. The gradual increase in the concentration of acid phosphatase correlated with an increase of its activity in the haemolymph of the inoculated larvae as a response to infection with *S. aureus*. Acid phosphatase, used as a marker enzyme for lysosomes and apoptosis, involved in the degradation of insect tissues [30]. Lysosomal participation during the programmed cell death of tissues has been confirmed by the elevated level of the acid phosphatase [31]. Variations in the activity of the acid phosphatase of silkworm were studied in all stages of the life cycle of the *Bombyx mori*, during spinning stage, a steady increase was recorded in the activity of acid phosphatase [32]. Acid phosphatase may have a role in autophagy and cell turnover as well as defense [33].

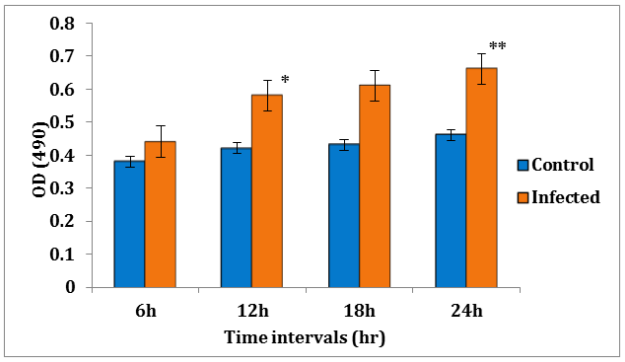

Figure 2: Phenoloxidase activity in the haemolymph of control and infected larvae of *B. mori* Values are mean ± SE from 8 silkworms in each group. Superscripts are significantly different at *P ≤ 0.05, **P ≤ 0.01.

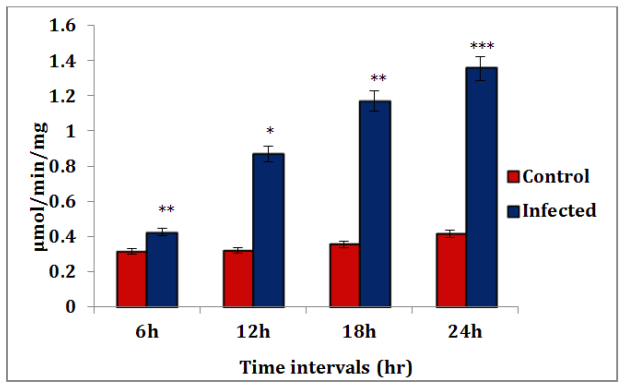

Figure 3: Acid phosphatase activity in the haemolymph of control and infected larvae of *B. mori* Values are mean ± SE from 8 silkworms in each group. Superscripts are significantly different at *P ≤ 0.05, **P ≤ 0.01, **P ≤ 0.01.

Estimation of protein in the haemolymph

Increased trend of protein concentration was recorded in infected group from 12, 18 and 24 hours post infection with *S. aureus* (Figure 4). The major changes were not observed at 6 hours post infection when compared with control group. The haemolymph composition of

insects reflects the nature and degree of metabolism of the tissues, any changes in the protein concentration of the haemolymph may show the level of modification in the organism [34]. During infection profound biochemical changes occur in the haemolymph, particularly in the concentration of proteins. The immune response induced against *S. aureus* in response to infection is the synthesis of antimicrobial peptides in silkworm haemolymph may be the reason for the elevation of protein content during bacterial infection.

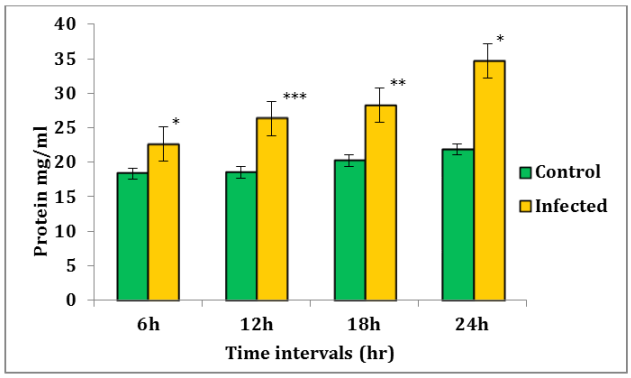

Figure 4: Protein concentration in the haemolymph of control and infected larvae of *B. mori* Values are mean ± SE from 8 silkworms in each group. Superscripts are significantly different at *P ≤ 0.05, **P ≤ 0.01, **P ≤ 0.01.

Glutathione peroxidase (GPx) assay

Glutathione peroxidase (GPx) activity decreased gradually in infected group when compared with control group. The lowest GPx activity (0.121 nmol NADPH/min/mg protein) was recorded at 24 hours post infection with bacteria (Figure 5). GPx is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. The biochemical function of GPx is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. Glutathione peroxidase activity inhibited loss of polyunsaturated fatty acids from microsomes and mitochondria when they were exposed to peroxidizing conditions [35]. Alterations of these enzyme levels are associated with diverse cancer types, including skin, kidney, intestine and breast cancer [36].

Catalase (CAT) assay

Cytosolic enzyme catalase is a component of antioxidant defense system that reduces hydrogen peroxide (H_2O_2) to water and protect the cell from oxidative damage [37]. A significant reduction in enzyme activity was noticed in the infected larvae when compared to control group after 24 hours of infection with pathogenic bacteria (Figure 6). The peroxisomal catalases might be crucial factors for adaptation to oxidative stress generated during bacterial growth [38,39]. The significant reduction of the enzyme activity was observed in infected larvae which may be due to metabolic adjustments as the part of the defense system of the host as a consequence of bacterial invasion.

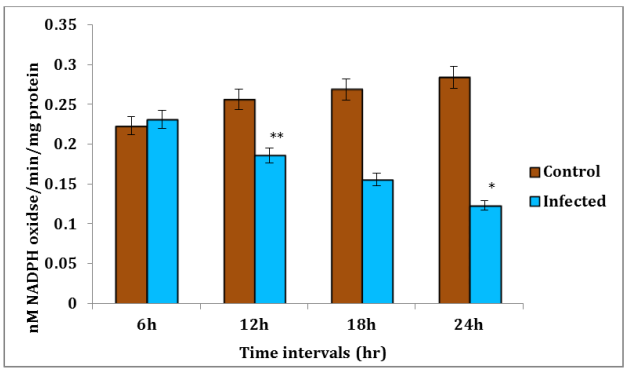

Figure 5: Glutathione peroxidase activity in the haemolymph of control and infected larvae of *B. mori*. Values are mean ± SE from 8 silkworms in each group. Superscripts are significantly different at *P ≤ 0.05, **P ≤ 0.01.

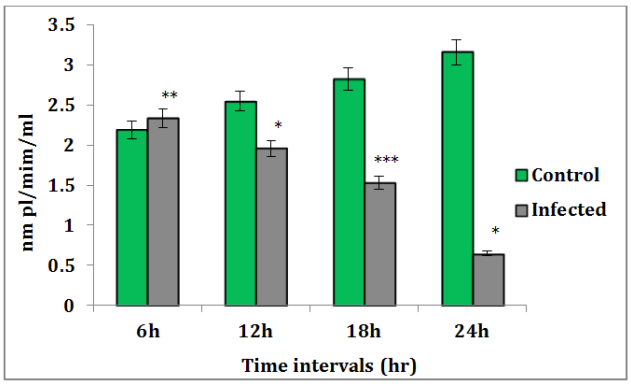

Figure 6: Catalase activity in the haemolymph of control and infected larvae of *B. mori*. Values are mean ± SE from 8 silkworms in each group. Superscripts are significantly different at *P ≤ 0.05, **P ≤ 0.01, **P ≤ 0.01.

Superoxide dismutase (SOD) assay

Superoxide dismutase (SOD) activity increased at 6 hours post infection, during the later hours of infection SOD activity decreased gradually when compared to control group (Figure 7). The highest SOD activity (0.325 U/mg protein) was recorded at 6 hours post infection and lowest activity (0.192 U/mg protein) at 24 hours post infection with bacteria. Superoxide dismutase (SOD) is an enzyme facilitating the removal of superoxide anions from living organisms, which catalyzes the conversion of the superoxide radicals into molecular O_2 and H_2O_2 and thus form a crucial part of the cellular antioxidant defense mechanism [40]. SOD activity was decreased in the haemolymph of infected larvae, due to the significant reductions in plasma paraoxonase activity, thiol concentrations in infected larvae.

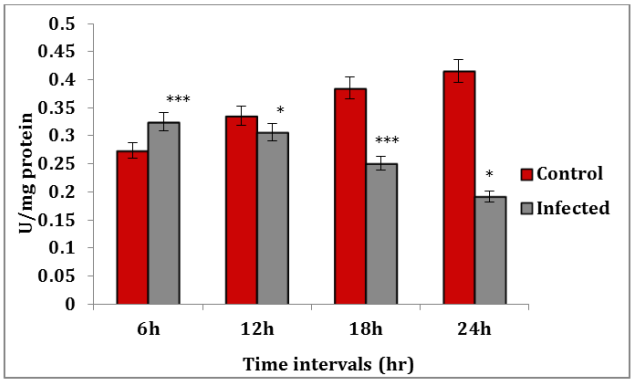

Figure 7: Superoxide dismutase activity in the haemolymph of control and infected larvae of *B. mori*. Values are mean ± SE from 8 silkworms in each group. Superscripts are significantly different at *P ≤ 0.05, **P ≤ 0.01, **P ≤ 0.01.

Mortality

In the developmental life cycle of an insect, considerable variation in the susceptibility has been established. Each stage differs in its susceptibility and degree of resistance. At 6 hours of infection with bacteria only 0% of mortality was reported, the percentage of mortality was gradually increased at 12, 18 and 24 hours of infection with bacteria (Figure 8). The percentage of mortality clearly demonstrates an increased susceptibility at 12, 18 and 24 hours of infection with bacteria.

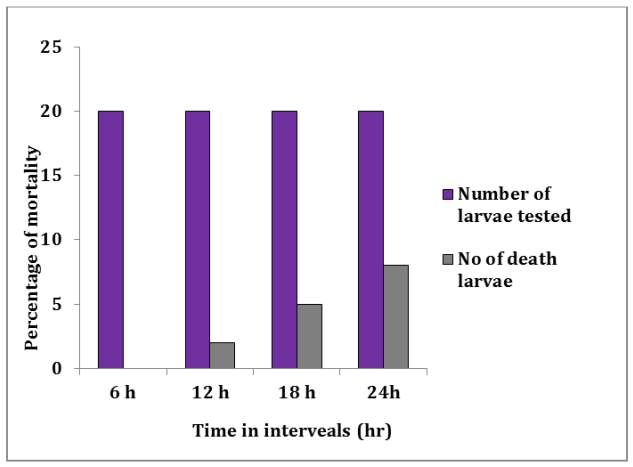

Figure 8: Relationship between *S. aureus* pathogenicity and silkworm larvae.

Measurement of silk gland weight

The major changes were not observed in the silk glands of infected group when compared with control group. But the weight of the silk glands in infected group was lower when compared with control group (Figure 9). At every hour silkworms were dissected and the silk glands (5 pairs) were collected, the weights of silk glands were recorded in grams. The silk glands do not exhibit any signs and symptoms of bacterial infection (Figure 10).

Silkworm salivary glands are not susceptible to bacterial and viral infections [41]. Silk gland weight and shell weight also significantly declined following infection over uninfected larvae. Silk gland assimilates the mulberry protein in the course of larval development and grows considerable size and weight to provide protein material during the spinning of the cocoon. The process of protein assimilation and growth of silk gland depend mainly on the physiological conditions of the silkworm during larval growth. The decrease in weight of the silk gland was observed in the infected larvae when compared with control group. This suggests that *S. aureus* interferes with the normal physiology of the silkworm and affects the weight of the silk gland possibly by reducing the assimilation of proteins.

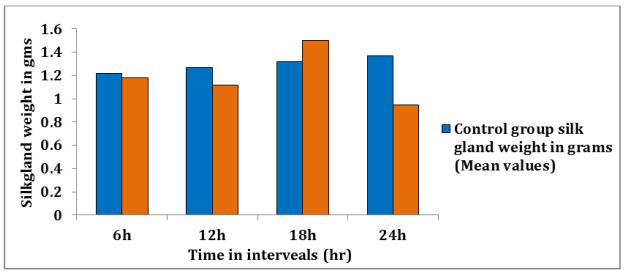

Figure 9: Measurement of silk gland weight of the control and infected larvae (Silk gland weight in grams (Mean values).

Figure 10: Pair of silk glands from (A). Control and (B). Infected silk worm larvae after 24 h infection with bacteria.

Conclusion

Infection of *S. aureus* causes significant increase in MDA concentration, acid phosphatase and phenoloxidase activity after 24 h of infection in fifth instar silkworm larvae. Protein concentration in haemolymph markedly increased in infected larvae following appearance of disease symptoms. *S. aureus* has ability to altering antioxidant levels such as superoxide dismutases (SOD), catalase and glutathione levels and cause death of silkworms. The silk glands did not exhibit any signs and symptoms of bacterial infection indicated that silk glands are resistant to *S. aureus* infection. From the above studies it is concluded that silkworms as a good animal model to study pathogenicity of bacteria.

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