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Studies on the haemolymph glucose level in silkworm *Bombyx mori* during cytoplasmic polyhedrosis

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ABSTRACT

Two mulberry silkworm breeds viz., Pure Mysore and NB_4D_2 were selected for the present study. The breed susceptibility was determined during fifth instar by oral injection method. Two doses of cytoplasmic polyhedral inclusion bodies i.e., 1.562 x 10^6 and 3.125 x 10^6 were selected based on the breed susceptibility test and inoculated in to silkworm larvae by oral injection. The control as well as inoculated silkworm larvae were collected daily with a regular interval of 24 h for haemolymph collection. The collected haemolymph was subjected for glucose assay. Among the experimental batches, the diseased larvae exhibited reduction in the blood glucose level and also there was gradual reduction as the disease progressed. Such changes in the infected larvae, depicting the possible defense cellular adjustment of the host in response to viral attack.

Key words: *Bombyx mori, cytoplasmic polyhedrosis, haemolymph, glucose level.*

INTRODUCTION

In the tissues of insects with an infectious disease, various biochemical, physiological and cytomorphological alterations can be observed [1, 2, 3, 4, 5, 6]. Most of the biochemical studies associated with cytoplasmic polyhedrosis in silkworm *Bombyx mori* are daily changes in the quantity of nucleic acid and protein in the blood and midgut [7]; activities of several enzymes responsible for carbohydrate metabolism in midgut epithelium of *Bombyx mori* [8]; amino acids decreased in the haemolymph and midgut epithelium [9]; enhanced protein metabolism in the midgut epithelium [10]; amylase and succinate dehydrogenase activity levels [5]; effects of cytoplasmic polyhedrosis on silkworm *Bombyx mori* L. and its transmission from generation to generation [11] and Effect of cytoplasmic polyhedrosis on haemolymph proteins of silkworm [4]. The analysis of biomolecules like proteins, amylase, succinate dehydrogenase [3, 11, 5], alkaline phosphatase and alkaline protease [12] and molecular marker assisted breeding [13] will help in the silkworm breeding programme for cocoon characters and disease resistance. However, studies combining biomolecules like glucose with cytoplasmic polyhedrosis are rather scarce. Hence, the present investigation was undertaken to study the effects of cytoplasmic polyhedrosis on blood glucose level.

MATERIAL AND METHODS

Two silkworm breeds namely, Pure Mysore and NB_4D_2 were selected as experimental system for the present investigation. The silkworm rearing was conducted in the laboratory following the method described by Krishnaswami [14]. The Cytoplasmic Polyhedral Inclusion Bodies (CPIBs), obtained from Sericulture Department, University of Agricultural Sciences, G.K.V.K. Bangalore, India, were *per orally* inoculated into the silkworm larvae immediately after second moult for the multiplication of virus. After 10 days of inoculation, the midgut of the worms which exhibited flaccid condition were dissected and the

white midguts were selected. Such midguts were used for the isolation of polyhedral inclusion bodies (PIBs). Purification was carried out by following the method described by Balakrishnappa and Honnaiah [15]. Finally, the stock suspension was prepared which contained 6.25 X 10^6 polyhedra per ml. Enumeration of polyhedra was done by following Neuber's haemocytometer.

To determine the dosage for inoculation, the susceptibility silkworm breeds was screened against *Bm*CPV, immediately after 4th moult, that is, after first two feedings during fifth instar. A total of seven batches, each batch containing 50 worms in triplicate, were inoculated *per oral* with 40µl of different concentrations of *Bm*CPV *viz*., 6.25 x 10^6, 3.125 x 10^6, 1.562 x 10^6, 0.781 x 10^6, 0.391 x 10^6, 0.195 x 10^6 and 0.0976 x 10^6 polyhedral bodies in 0.75% NaCl solution. The control worms received the same amount of 0.75% NaCl solution only. Later, the worms were allowed to complete larval stage, spinning, pupation and moth emergence. Number of cocoons harvested from each batch was considered for calculation of the breed susceptibility. Based on the results of breed susceptibility experiment, the silkworm larvae immediately after 4th moult, that is, after first two feedings during fifth instar were inoculated with two different doses of *Bm*CPV *viz* 1.562 x 10^6 (T_1 batch) and 3.125 x 10^6 (T_2 batch) by oral injection, and allowed to continue larval development. Each batch was consisted of 100 worms. Such silkworm larvae were collected daily with a regular interval of 24 hours from the time of inoculation till the end of fifth instar, and used for the estimation of blood glucose levels. The larvae from second day of fifth instar were collected daily with a regular interval of 24h till the end of fifth instar. The abdominal legs were punctured and haemolymph was collected in a pre cooled micro centrifuge tubes containing 1 mM thiourea as described by Mahesha *et al.* [16], centrifuged at 3000 rpm for 5 minutes in a cooling centrifuge at 5°C and preserved in a deep freezer at -20°C as stock and it was used whenever required. Protein free filtrate was prepared as described by Oser [17]. Blood glucose level was estimated by following the method of Folin-Wu as described by Oser [17]. The blood glucose level was expressed as mg glucose per ml haemolymph.

The data obtained from the biochemical experiments were statistically analyzed through SPSS by two way ANOVA, to determine the level of significance between experimental sets, between age groups and the interaction effect between the experimental sets and age groups [18], Scheffe's post hoc test [19] and Probit analysis [20] wherever they were applicable.

RESULTS AND DISCUSSION

The LC_{50} value for mortality was 0.096 x 10^6 PIB/ml in Pure Mysore followed by NB_4D_2 (0.218 x 10^6 PIB/ml). This indicates that Pure Mysore was more sensitive to *Bm*CPV when compared with NB_4D_2 race (Table 1). In Pure Mysore race, the viability rate was higher in the control batches and gradual reduction was noticed as the dose of *Bm*CPV increased. The control (C_O) worms recorded a mean viability of 92% followed by C_1 of 44%, C_2 of 36%, C_3 of 26%, C_4 of 24%, C_5 of 20%, C_6 of 14% and C_7 of 9.33% . The C_1, C_2, C_3, C_4, C_5, C_6 and C_7 are the silkworm batches inoculated with *Bm*CPV doses of 0.098 x 10^6, 0.195 x 10^6, 0.391 x 10^6, 0.781 x 10^6, 1.562 x 10^6, 3.125 x 10^6 and 6.25 x 10^6 PIB/ml (polyhedral inclusion bodies per ml) respectively. The variation among the experimental batches is found to be statistically significant at 0.0000 level. Further, the results of Scheffe's post hoc test revealed that the difference between the control and *Bm*CPV inoculated batches as well as among *Bm*CPV inoculated batches (except between C_3 and, C_4 and C_5 sets) is significant at 0.05 level. In the present investigation, breed susceptibility was carried out by oral inoculation technique. This method gives more accuracy in the entry of pathogen. Of the tested breeds, Pure Mysore, a multivoltine had LC_{50} value of 0.096 x 10^6 PIBs/ml and NB_4D_2 a bivoltine exhibited less susceptibility and the LC_{50} value of 0.218 x 10^6 PIBs/ml. Bhaskar [21] also reported Pure Mysore is sensitive in early stages of larval development. However, contrary to the general trend, the multivoltine Pure Mysore was found to be more sensitivity to *Bm*CPV as compared to a bivoltine. Such a contradiction might have direct relationship to the body weight as well as

larval duration that is a lighter race (Pure Mysore) is more susceptibility to *Bm*CPV as against the heavier race (NB_4D_2).

The blood glucose levels in Pure Mysore and NB_4D_2 larvae are shown in tables 2 and 3 respectively. The level of blood glucose in the control set of Pure Mysore larvae showed a significant drop during 5^{th} instar except on 6^{th} and 7^{th} day. The glucose level was increased by about 13.47% and 28.79%on 6^{th} and 7^{th} day respectively when compared to their respective previous day. The *Bm*CPV inoculated worms also followed the same pattern, but, with decreased amount of glucose. When the average blood glucose level during 5^{th} instar was taken, control worms showed 2.99 mg/ml, followed by T_1 of 2.54 mg/ml and T_2 of 2.4 mg/ml.

In case of control as well as *Bm*CPV infected NB_4D_2 larvae, the blood glucose level is significantly different from that of Pure Mysore larvae. In the control, the glucose level is gradually increased from the beginning to the end of fifth instar. In case of *Bm*CPV infected batches, both T_1 and T_2 sets showed the same pattern of glucose level as in the case of control batch. A well defined change in the quantity of glucose could be observed between control and diseased worms as well as among the infected batches. Of all the batches tested, the average blood glucose level during the fifth instar was found to be the highest in the control with 3.01 mg/ml followed by T_1 set with 2.6 mg/ml and T_2 set with 2.41 mg/ml. The results of two way ANOVA revealed that the variation between the tested batches, between age groups and the interaction effect between tested batches and age groups are all significant at 0.000 level. Higher blood glucose level observed in control batches, might reflect the higher rate of food ingestion and digestion. The drop in the blood glucose level of infected worms represents sustained depletion of the same since the CPV alters the normal function of the midgut. Apart from our observations, Kadoya *et al*. [22] also reported the sugar concentration in haemolymph was distinctly decreased in CPV infected larvae when compared to control. However, the pattern was similar to that of starved larvae. Their results also suggested that glycogen metabolism in midgut epithelium is affected more directly by CPV multiplication. Hence, the present investigation clearly indicated that the *Bm*CPV inoculated silkworms showed quantitative variation in haemolymph glucose level reflects the utilization of less food material, reduced rate of conversion and metabolism resulting in less production in the surviving silkworms as the main portion of the digestive system *i.e*., midgut is infected by virus. Such a basic knowledge about these biochemical aspects during cytoplasmic polyhedrosis is essential to plan detailed studies at the molecular level for identification of biochemical markers useful for developing disease resistant breeds. Also, the information obtained from this research work contributes a lot to basic virology in general.

Table 1: Effect of *Bm*CPV infection on larval mortality

Dose of *Bm*CPV	Pure Mysore Viability in %	NB_4D_2 Viability in %
C_0	92	90
C_1 (0.098×10^6)	44	52
C_2 ($0.0.195 \times 10^6$)	36	38
C_3 (0.391×10^6)	26	30
C_4 (0.781×10^6)	24	28
C_5 (1.562×10^6)	20	24
C_6 (3.125×10^6)	14	18
C_7 (6.25×10^6)	9.33	12

The variation between experimental sets is found to be statistically significant at 0.000 level.

Table 2: Amount of Glucose in haemolymph during fifth instar of Pure Mysore larvae treated with *BM*CPV (Values expressed as mg/ml)

Dose of *Bm*CPV	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day	8th Day	Average concentration
Control	4.890	3.460 (-25.56)	2.665 (-26.78)	1.974 (-25.93)	2.240 (+13.47)	2.885 (+28.79)	2.810 (-2.56)	2.989
T_1 (1.562×10^6)	4.740 (-3.07)	3.380 (-28.69) (-7.14)	2.110 (-37.57) (-20.83)	1.530 (-27.48) (-22.49)	1.980 (+29.41) (-11.61)	2.090 (+5.55) (-27.56)	1.921 (-8.090) (-31.64)	2.536
T_2 (3.125×10^6)	4.510 (-7.77)	3.260 (-27.72) (-10.44)	2.040 (-37.42) (-23.45)	1.440 (-29.41) (-27.05)	1.720 (+19.44) (-23.21)	2.010 (+16.86) (-30.33)	1.839 (-8.51) (-34.55)	2.402

Values within parenthesis (1st row) represents percent change over previous day.
Values within parenthesis (2st row) represent percent change over control.
The variation between experimental sets, between age groups and the interaction effect between experimental sets and age groups are all found to be statistically significant at 0.000 level.

Table 3: Amount of Glucose in haemolymph during fifth instar of NB_4D_2 larvae treated with *BM*CPV (Values expressed as mg/ml)

Dose of *Bm*CPV	2nd Day	3rd Day	4th Day	5th Day	6th Day	Average concentration
Control	1.025	2.230 (+117.56)	2.489 (+11.61)	3.067 (+23.22)	6.257 (+104.01)	3.014
T_1 (1.562×10^6)	1.071 (-0.78)	2.073 (+103.83) (-7.04)	2.117 (+2.12) (-14.95)	2.67 (+26.12) (-12.94)	5.080 (+90.26) (-18.81)	2.602
T_2 (3.125×10^6)	1.008 (-1.66)	1.930 (+91.47) (-13.45)	2.010 (+4.15) (-19.24)	2.250 (+11.94) (-26.64)	4.870 (+116.44) (-22.17)	2.413

Values within parenthesis (1st row) represents percent change over previous day.
Values within parenthesis (2st row) represent percent change over control.
The variation between experimental sets, between age groups and the interaction effect between experimental sets and age groups are all found to be statistically significant at 0.000 level.

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