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The Effects of Vitamin C on Biological, Biochemical and Economical Characteristics of the Silkworm, *Bombyx mori* L.

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In order to investigate the effects of supplementary nutrients on silkworms, *Bombyx mori*, an experiment was conducted with ascorbic acid treatments. Dietary supplements of ascorbic acid 1, 2 and 3% were fed to silkworm larvae through 1st to 5th instar. The larvae were fed by mulberry leaves of Kokoso variety and the supplementary leaves were used once a day. These treatments resulted in a significant increase of biological parameters such as larval weight, the rate of food consumption and the approximate digestibility of the food. But the economical parameters such as cocoon weight and cocoon shell weight didnt show considerable difference compared to control. Dietary supplement of 2% ascorbic acid increased the larval weight by 7.8% and reached to 1.065 g, which had the highest weight increase in the fourth day of 4th instar larvae. The percentage of daily weight increase in this group of larvae (79.01%) had significant difference compared with other treatments. The nutritional efficiency index in this group of larvae was better than others. Also the abundance of biochemical macromolecules such as glucose, cholesterol, triacylglycerol and urea in haemolymph of larvae fed by 2% ascorbic acid increased to become 29.75 (mg/dl), 24 (mg/dl), 75.4 (mg/ml) and 32.1 (mg/ml) respectively. But protein contents of haemolymph of larvae in each treatment were not significantly different. Since all the results achieved were not considerable either statistically or economically, this method could not be recommended to improve the sericultural parameters.

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Introduction

The production of good quality and quantity of silk depends on healthiness of larva and larval nutrition, which are partially influenced by the nutritive value of mulberry leaves (Ito, 1978). The silkworm requires certain essential sugars, proteins, amino acids and vitamins for its normal growth, survival and enhancement of the silk production. The silkworm *Bombyx mori* mainly depends on mulberry leaves for its vitamin requirement since it is a monophagous insect (Ito, 1978; Horie, 1995).

It is generally accepted that all insects require vitamins, especially water-soluble vitamins such as ascorbic acid, thiamin, riboflavin, niacin, pyridoxine, pantothenic acid, biotin, folic acid and choline (Chapman, 1998). Growth retardation caused by lack of each one of these vitamins is rather small, but better growth is obtained by adding these vitamins to their diet (Horie and Ito, 1965; Ito, 1978; Horie, 1995). The nutritional status of the mulberry leaves can be improved by enriching them with vitamins and other nutrients. Fortification of mulberry leaves with complementary compounds was found to increase the larval growth and post cocoon characteristics (Das and Medda, 1988; El-Karaksy and Idriss, 1990; Lalita *et al.*, 1991; Muniandy *et al.*, 1995; Sarker *et al.*, 1995; Nirwani and Kaliwal, 1996; Etabri, 2002, Etebari and Fazilati, 2003).

Muniandy *et al.* (1995) showed that multivitamins and mineral compounds could increase the food intake, growth and conversion efficiency in silkworm. Nirwani and Kaliwal (1996) showed that folic acid causes a significant increase in economical parameters such as female and male cocoon weight, shell weight and egg production.

Also the dietary supplementation with B-complex to silkworm larvae resulted in a significant increase of female fecundity and improved cocoon yield and filament length of the silk (Sarker *et al.*, 1995).

Ascorbic acid ($C_6H_8O_6$) is the active form of vitamin C and is a water soluble vitamin. It has been found in different insects tissues and organs. The function of vitamin C is not fully known yet but it is assumed that like mammals, it involves in tyrosine metabolism, steroids synthesis, carnetin synthesis, norumodealutory, feeding stimulatory, immunity and detoxifications. Therefore, the necessity of this compound in the diet of herbivorouse insects for feeding requirement and antioxidant activity has been confirmed by authors (Felton and Summers, 1993, 1995).

It is assumed that ascorbic acid has an important role on ecdysis and formation of cuticle (Navon, 1978; Dong and Zheng, 1984; Lindroth and Weiss, 1994). Matsuda (1981) reported that ascorbic acid increases feeding activity in some coleoptera. Other authors have introduced ascorbic acid as a feeding stimulatory factor on *Achoea janata* (Singh and Reddy, 1981). But Allsopp (1992) reported that this vitamin did not stimulate the feeding of 3rd instar larva of *Antitrogus parvulus*. Others had mentioned such characteristics of vitamin C and even had suggested that the vitamin prevents the feeding of *Trichoplusia ni* (Gothif and Beck, 1967).

With respect to what was said about different suggestions on ascorbic acid effects and taking in mind that the abundance of vitamin C in mulberry leaves has been reported by several authors, but the quantity of this vitamin is very variable in different conditions (Ito, 1978; Babu *et al.*, 1992), the necessity of ascorbic acid in insects diet becomes clarified. Authors believe that the hosts of individual insects supply most of its nutritional needs, but could this always be true?

Therefore, the present investigation was undertaken to study the effects of ascorbic acid on the biological and economical parameters of Iranian hybrid of silkworm.

Materials and Methods

Rearing

The eggs of bivoltine hybrid silkworm (103 × 104) were obtained from Iran Silkworm Rearing Co. (Rasht, Iran), and reared in the laboratory with standard rearing technique (Lim *et al.*, 1990) under 25°C with RH of 75 ± 5% and photoperiod of 16L:8D. The larvae were fed by mulberry leaves of kokoso variety up to the last instar.

Treatments

First instar larvae were divided into 5 experimental groups, including 2 controls (normal and distilled water control). Each group consisted of 100 larvae with three replications. L(+) Ascorbic acid (Merck Co., Germany) was dissolved in distilled water and diluted to 1, 2 and 3% concentrations. Fresh mulberry leaves were soaked in each concentration for 15 min and then were dried in air for 20 min. The supplementary leaves were fed to silkworm from 1st to 5th instar, once a day.

Biological parameters

After the treatments, the larval and cocoon parameters were observed. The weight of larvae were determined by weighing 30 larvae in different days of fourth and fifth instars and the percentage of daily increase of weight (DIW%) was calculated for each group. Cocoon weight, cocoon shell weight, cocoon shell ratio and pupal weight were determined by using standard technique in sericulture that was described by Nirwani and Kaliwal (1996).

Feeding rates

In order to determine the last instars nutritional efficiency, the indices were calculated on the basis of procedures designed by Waldbauer (1968) on dry weight basis. The rate of food consumption [$CR = I/D.T$], which is the mean weight of dry food consumed per larva per day, I, an index was obtained by subtracting the oven dry weight of leaf fragment from the predicated oven dry weight of the remained leaf and accumulating these differences over the whole feeding period. Where D, is the density of larva in each replication and T, is the eating period of larva in last instar. The rate of feces production: [$FR = F/D.T$], which is the mean dry weight of feces produced per larva per day. The approximate digestibility of the food consumed: [$\%AD = 100(I - F)/I$], which slightly underestimates the fraction of the ingested food which is assimilated. The relative growth rate was [$GR = G/W.T$], where G, the fresh weight gain was computed as the difference between the initial and final weight of larva in each replication, W, the mean weight of the last instar feeding period. Also, in this process the efficiency of conversion of ingested food to body substance [$\%ECI = 100G/I$] and the efficiency of conversion of digested food to body substance: [$\%ECD = 100G/(I - F)$], were measured.

Oven dry weights of the feces and the remaining leaf fragments were obtained by drying them at 105°C for 24 hrs, then to cool down the materials, they were put in a descicator before weighing. To obtain dry weight of leaves before feeding, fresh leaves were used to obtain a regression relationship between oven dry weights as the dependent variable and fresh weight as independent variable. The best predictor of oven dried leaf weight (Y) was: $Y = 0.2279 X + 0.0854$ ($R2 = 0.94$), where X is fresh leaf weight.

Biochemical parameters

Haemolymph Preparation: Ten random samples from 5th instar larva in their 6th day of each replication were taken and one of their prolegs was cut. The haemolymph was collected in eppendorf microtubes and immediately phenylthiourea was added for preventing melanization. The samples were centrifuged at 14,000 rpm for 10 min. The supernatant was removed and kept in −20°C for analysis.

Analysis: Protein was measured by total protein assay kit (Darmankave Co., Iran) with bovine serum albumin as the standard protein. Uric acid contents in the heamolymph were determined using uricase as described by Valovage and Brooks (1979). The concentration of urea was determined by measuring ammonia produced from urea. This method utilizes enzymatic hydrolysis of urea followed by a colorimetric determination of resultant ammonia by using commercial urea assay kit (Chemenzyme Co., Iran). Glucose concentration of heamolymph was determined by hexokinase method of Siegert (1987). For total cholesterol assessment of haemolymph the method described by Richmond (1973) was used. The basis of this method is on the hydrolysis of cholesterol ester by cholesterol oxidase, esterase and proxidase enzymes. Triacylglycerol of haemolymph was measured utilizing Buclo and Davids methods (1973), which was also based on the hydrolysis of haemolymph triacylglycerol by enzymes such as lipase, glycerokinase, glycerol-3-phosphate oxidase and proxidase. Finally the measurement of released glycerol by colorimetric in 520 nm was used by spectrophotometer.

Statistical calculations

Collected data were subjected to statistical analysis of variance test to find out the low significant different between the parameters of the normal control and treated groups. For all analysis of variance the Duncans multiple rang test in SAS software was used (Sas, 1997).

Result

The effects of dietary supplementations of ascorbic acid on some biological, economical and biochemical parameters of silkworm are presented in the Table 1 to 4.

Biological and economical parameters

Larval weight: The larval weight significantly increased in all treated groups (Table 1). The larvae under 1% treat-

Table 1. The effects of ascorbic acid on the larval weight (g) of silkworm

Concen. (%)	4th Instar		5th Instar			
	1st day	4th day	1st day	3rd day	5th day	7th day
1	0.1838a (106.6)[1]	1.052a (106.4)	1.020ab (102.1)	2.423c (94.3)	4.356ab (103.2)	5.547b (98.7)
2	0.1820a (105.6)	1.065a (107.8)	1.041a (104.2)	2.589b (100.8)	4.343ab (102.9)	5.782a (102.9)
3	0.1745bc (101.2)	1.020ab (103.2)	0.970c (97.1)	2.418c (94.1)	4.321ab (102.4)	5.610ab (99.8)
C	0.1723c (100)	0.988b (100)	0.999bc (100)	2.568b (100)	4.218b (100)	5.619ab (100)
DWC	0.1793ab (104.6)	0.994b (100.6)	0.988bc (99.9)	2.632a (102.4)	4.409a (104.5)	5.585ab (99.3)

C: normal control, DWC: distilled water control.

[1]The percentage of difference between control and treatments.

Significant at $\alpha = 5\%$.

Table 2. The effects of ascorbic acid on the cocoon parameters of silkworm

Concen (%)	Female				Male			
	Cocoon weight (g)	Pupa weight (g)	C. shell weight (g)	C. shell ratio (%)	Cocoon weight (g)	Pupa weight (g)	C. shell weight (g)	C. shell ratio (%)
1	3.037a (96.8)[1]	2.317a (96.2)	0.721ab (98.9)	23.77a	2.355a (100.1)	1.687a (103.4)	0.667 b (95.9)	28.92ab
2	3.126a (99.6)	2.381a (98.9)	0.744 a (102.1)	23.83a	2.417a (102.8)	1.713a (105.1)	0.705 a (101.4)	29.27 a
3	3.030a (96.6)	2.301a (95.5)	0.725ab (99.4)	24.00a	2.375a (100.9)	1.662a (101.9)	0.702 a (101.0)	29.72 a
C	3.136a (100)	2.407a (100)	0.729ab (100)	23.32a	2.352a (100)	1.631a (100)	0.695ab (100)	29.57 a
DWC	3.091a (98.5)	2.382a (98.8)	0.708 b (97.1)	22.96b	2.357a (100.2)	1.697a (104.1)	0.663 b (95.3)	28.27 b

C: normal control, DWC: distilled water control.

[1]The percentage of different between control and treatment.

Significant at $\alpha = 5\%$.

ment had 6.6% weight increase compared to normal control but they showed no significant difference between 1 and 2% concentrations.

Three percentage concentration of ascorbic acid didnt show any statistical difference with control either. These weight variations at the 4th day of fourth instar in 2% concentration reached its maximum and the larvae of this group with 7.8% increase, gained 1.065 g weight. The larval weight in 2% concentration during 5th instar was relatively good but its most highest weight increase was 4.2% at the 1st day. At 5th instar, the increase in the weight of larva was not the same as what we expected to have, *i.e.*, the increase of weight equivalent to whatever we obtained in 4th instar. The larval weight in distilled water treatment was more than control throughout 5th instar.

Cocoon characteristics: As shown in Table 2, the cocoon weight not only hasnt increased but also it relatively decreased. Consequently the pupal weight in this group had the same situation. While the cocoon shell weight of females in 2% concentration with more than 2.1% weight increase was relatively better than control, this increase isnt statistically significant. Males also followed the same changes. The male pupal weight in 2% concentration shows 5% increase, but there is no significant difference between the cocoon weight and pupal weight in the treatments.

Nutritional efficiency indices: The effects of ascorbic acid on nutritional efficiency indices of 5th instar larvae are given in Table 3. The daily consumption rate of each larva in distilled water treatment is more than other treatments. Ascorbic acid has caused better food intake and 2% concentration has the highest value. The most important use of FR is in calculating the AD. Where control larvae had much more FR, the approximate digestibility of food index in this group has significantly decreased.

Table 3. The effects of ascorbic acid on 5th instar larval nutritional efficiency index

Concen. (%)	CR	FR	AD%	GR	ECI%	ECD%
1	0.770 c	0.318cd	58.6 c	0.816 a	2.10 b	3.57 b
2	0.820 b	0.332 c	89.6 a	0.819 a	2.05 b	3.44 b
3	0.780 c	0.306 d	60.9 b	0.827 a	2.11 b	3.47 b
C	0.704 d	0.521 a	26.1 d	0.822 a	2.34 a	9.00 a
DWC	0.852 a	0.352 b	58.7 c	0.823 a	1.92 c	3.28 b

C: normal control, CR: Consumption rate of food, DWC: distilled water control, FR: Feces production rate, AD: Approximate digestibility of food, GR: Growth rate, ECI: Efficiency of conversion of ingested food, ECD: Efficiency of conversion of digested food.

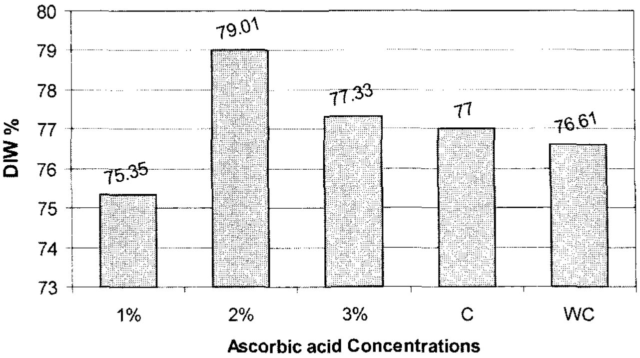

Fig 1. The effects of ascorbic acid on 5th instar larval daily increased weight.

The FR in different concentrations and distilled water is less than control, so AD in all treatments was more than the control. The larva fed on 2% concentration of ascorbic acid with 89.6% had the highest AD. The growth rate of larvae in all treatments, except little differences, showed no statistical differences. But the percentage of daily increased weight in 2% concentration with 79.01% compared to 77% of control is relatively better than others (Fig. 1). Two important factors, efficiency of conversion of ingested food and efficiency of conversion of digested food, in control were more than other groups.

Biochemical parameters

Glucose in larvae treated by 2% concentration of ascorbic acid shows significant increase and reaches to 29.750 mg/dl (Table 4). The amount of glucose in the haemolymph of larvae treated with 3% concentration of ascorbic acid was significantly lower (14.50 mg/dl) than other treatments and the control. Results showed that feeding on leaves supplemented by ascorbic acid doesnt have any effect on the total protein of haemolymph. Urea in 5th instar larvae treated by 3% concentration of ascorbic acid and distilled water was lower than control but the rest of treatments didnt show significant difference with each other but were significantly higher than control. Also uric acid in silkworm haemolymph is always less than urea. The variations of this compound in 5th instar larvae showed relative decrease compared to control. As is shown in Table 4, this amount is considerably decreased in 3% concentration (1.59 mg/dl).

Triacylglycerol in 2% ascorbic acid was 75.4 mg/ml, which is significantly more than control but there are no significant difference between 1 and 3% concentrations, although their amounts are relatively higher than controls. Cholesterol didnt change through different concentrations, but 3% concentration with 19.10 mg/dl, showed considerable decrease.

Table 4. The effects of ascorbic acid on biochemical macromolecules of larval haemolymph

Concen. (%)	Glucose (mg/dl)	Protein (g/dl)	Urea (mg/ml)	Uric acid (mg/dl)	Triacylglycerol (mg/ml)	Cholesterol (mg/dl)
1%	18.25 b	8.9 a	32.0 a	2.28ab	64.8 b	22.75ab
2%	29.75 a	9.6 a	32.1 a	2.85ab	75.4 a	24.00 a
3%	14.50 c	7.2 a	27.2 c	1.09 b	68.6 b	19.10 b
C	18.12 b	9.5 a	30.8 b	3.92 a	45.0 c	24.25 a
DWC	20.00 b	8.6 a	26.5 c	3.02 a	47.5 c	23.37 a

C: normal control, DWC: distilled water control.

Discussion

The most important economical factors in sericulture are the cocoon and its shell weights. These treatments couldnt increase these factors. Previously Etebari (2002) showed that although the cocoon weight has increased, the shell weight enhancement is only 1%, which is not statistically significant. Evanglista *et al.* (1997) also reported that the larval and cocoon weight increase under multi vitamin treatment hasnt any effects on the cocoon shell weight. It appears that this subject has some relations with the amount of protein in haemolymph. When treated the silkworm larval diet by folic acid, Nirwani and Kaliwal (1996) found that it increased the larval, cocoon and cocoon shell weight and they showed that the haemolymph protein in these group of larvae was more than control.

Some authors have studied the effects of different concentrations of ascorbic acid on wide range insects such as silkworm and various results have been achieved (Gothif and Beck, 1967; Singh and Reddy, 1981; El-Karkasi and Edris, 1990; Etebari, 2002). But there are no available articles on the effects of this vitamin on biochemical parameters of insects haemolymph.

Previously it was reported that when 5th instar larvae of silkworm feed on leaves supplemented by 0.25 – 2% concentration of ascorbic acid significant increase is observed on larval and pupal weight, whereas the most weight increase has occurred in 2% concentration. In recent research 2% concentration had also relatively good effects. This group of larva had the most larval weight and the DIW% was also higher than other treatments. As mentioned before these larvae had consumed more food and their AD index had reached 89.6%. As a consequence of more feeding the haemolymph glucose was higher and therfore they had higher performance.

The amount of glucose in haemolymph can be a representative aspect of carbohydrate metabolism. Although trihalose is the main haemolymph sugar in the most of insects but measuring haemolymph glucose as a cellular available sugar is valuable. Satake *et al.* (2000) showed that the quality of the food taken by larvae would have considerable effect on the haemolymph glucose.

Its been reported that with improving the biological performance of silkworm larvae treated with folic acid, the amount of haemolymph trihalose increased. Nirwani and Kaliwal (1996) suggested that this enhancement was related to phagostimulation of folic acid. Several authors also reported this effect about ascorbic acid (Dobzhenok, 1974; Matsuda, 1981; Singh and Reddy, 1981; Ito, 1987; El-Karkasy and Edris, 1990). Increasing the larval weight, DIW%, CR, AD and as a result the better absorption of many important biochemical compounds in 2% treatment of ascorbic acid, beside the phagostimulation characteristics of this compound can be due to the antioxidant role of this vitamin (Felton and Summers, 1993, 1995; Timmermann *et al.*, 1999). The antioxidant activity of ascorbic acid decreases the reactive oxygen species and oxidative pressure, as a result the absorption of the nutrients in the midgut would increase (Felton and Duffey, 1992; Felton, 1995; Barbehen *et al.*, 2001).

In recent research as AD in different treatments rised, the ECD index decreased. Volney *et al.* (1983) reported that there is a negative correlation between AD and ECD in California oakworm. Roth *et al.* (1994) determined that although AD index in gipsy moth *Lymantria dispar* has increased, it hasnt changed ECD index due to the special diet. Muniandy *et al.* (1995) also confirmed these results. In 3% concentration of ascorbic acid, some biological characteristics such as larval weight has decreased which appears to be due to hypervitaminose. Extended amounts of vitamin in insects diet also have negative effects and decrease the feeding. Previously Etebari (2002) reported these effects from niacin on silkworm.

The changes of urea concentration in the haemolymph are related to different factors like the age and diet of larva (Sumida *et al.*, 1993). These changes have direct relations to nitrogen and amino acids metabolism (Sumida *et al.*, 1993; Hirayama *et al.*, 1999; Dungern and Briegel, 2001). The feeding enhancement in larvae treated by 1 and 2% concentrations may be the main reason for the enhancement of haemolymph urea. This may be due to the increased metabolism as a result of increased intake.

With respect to the results of this study and other articles, the most effective factors on having various results are the treated instar and the mulberry leaves variety and also the climate. The results achieved show this system doesnt answer the economical needs for increased production of sericulture.

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