Effects of hypervitaminosis of vitamin B_3 on silkworm biology

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A high-dose of vitamin B_3 in silkworm diet interrupts larval feeding and normal growth. High mortality of larvae occurs during molting and they cannot complete this process normally. Also the larvae exhibit nicotinamide hypervitaminosis symptoms such as immobility, dyspepsia, darkening of the skin, inability to excrete normally, exerting brownish fluid from anus and swelling of rectal muscles. Maximum larval weights in 1, 2 and 3 g/l treatments were 2·9, 1·6 and 1·2 g respectively, while maximum larval weight in the control was 5·6 g. Larval stage compared to control had increased 18, 26 and 31 days respectively. The concentration increase of uric acid in haemolymph demonstrates the hyperuricemia, while other measured biochemical compounds show significant decrease; sodium and potassium did not change significantly.

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1. Introduction

Recently, much research has been done on the diet supplementation of mulberry leaves fed to silkworms. These supplementations include vitamins such as ascorbic acid, thiamin, niacin, folic acid and multi-vitamins (Etebari 2002; Nirwani and Kaliwal 1996, 1998; Saha and Khan 1996; Etebari *et al* 2004). Although some of the compounds have shown significant results, enrichment has not always caused the improvement of biological characteristics of the silkworm. In different studies, increase of supplement compound or increase of the period of its use show negative effects that could be due to hypervitaminosis.

Etebari *et al* (2004) reported the yield decrease, when ascorbic acid concentration is enhanced in silkworm diet. Saha and Khan (1996) reported the same effects from multi-vitamins. When silkworm larvae were treated with different concentrations of nicotinamide, high mortality was observed, although this vitamin is essential for *Bombyx mori* (Etebari 2002). Dosage of this vitamin is very determinative for normal growth of Mediterranean fruit fly. Chang and Li (2004) reported that nutritional interactions exist between vitamin B_3 and other groups of vitamin B.

Vitamin B_3 (niacin) comes in two forms, nicotinic acid and nicotinamide. Either form can satisfy the requirement for this vitamin in insects. Niacin, required for cell respiration, helps with the release of energy and metabolism of carbohydrates, lipids, and proteins vitamin B_3 is essential in the form of the coenzymes nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP) in which the nicotinamide moiety acts as an electron acceptor or hydrogen donor in many biological redox reactions (Alhadeff *et al* 1984; Swendseid and Jacob 1994; Spallholz *et al* 1999).

Little research has been done on the role of this vitamin in insects. The presence of this vitamin for appropriate growth of larvae and the reproduction in many insects and also mites has been reported (Ishii 1971; Yazgan 1972; Baker 1975; Jang 1986; Ritter and Johnson 1991; Levinson *et al* 1992; Ozalp and Emre 1992; Chang and Li 2004). Jang (1986) reported that the elimination of niacin (nicotinic acid) from *Ceratitis capitata* diet causes the increased mortality of larvae and decrease in the proportion

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Abbreviations used: DIW, Daily increase of weight; NAD, nicotinamide adenine dinucleotide; NADP, NAD phosphate.

of pupal to adult emergence. Deficiency of vitamin B_3 in the diet structure does not improve by adding tryptophan, but nicotinamide, NAD and NADP are proper substitutes for resumption of normal larval growth.

Nicotinic acid is often essential for oviposition of insects and it has been reported that removing nicotinic acid from diet of parasitoid *Pimpla turionellae* greatly reduces oviposition of this insect (Ozalp and Emre 1992). Requirements for this substance are very important but the question whether the enrichment of diet could increase the biological performance of insects has never been considered.

Supplement studies in flour beetles (*Tribolium confusum*) found that the enhancement with niacin failed to increase the oviposition rate considerably (Hamalainen and Loschiavo 1977). It is known that their normal food has a sufficient amount of this vitamin to meet their limited needs.

Different studies have reported that the amount of vitamin B_3 in mulberry leaves is 69–99 mg per each gram of dry weight and the minimum essential amount is 20 mg per each gram of dry weight of diet (Horie and Ito 1965; Ito 1978). Lots of researches suggest that different factors could contribute to the deficiency of the nutritients in mulberry leaves and consequently they considered supplementation (Ito 1978).

There are few studies on the effects of hypervitaminosis on insects. This research investigates the effects of vitamin B_3 enhancement in the diet of silkworm as a biological model.

2. Materials and methods

2.1 *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 mulberry leaves of kokoso variety up to the last instar.

2.2 *Treatments*

Fourth instar larvae were divided into 5 experimental groups including 2 controls (normal and distilled water control) each group consisting of 100 larvae. Nicotinamide ($C_6H_6N_2O$, Merck, Germany) was dissolved in distilled water and diluted to 1, 2 and 3 g/l concentration. Fresh mulberry leaves were soaked in each concentration for 15 min and then were dried in air for 10 min. The supplementary leaves were fed to silkworms from 4th to 5th instar, once a day. Therefore the silkworm larvae were fed supplementary leaves just seven times (3 times in 4th instar and 4 times in 5th instar).

2.3 *Biological parameters*

Post-treatment, the larval and cocoon parameters were observed. The weights of larvae were determined in different days (1st, 3rd, 5th and 7th) and the percentage of daily increase of weight (DIW) was calculated for each group. Cocoon weight, cocoon shell weight and pupal weight were determined (Nirwani and Kaliwal 1996).

2.4 *Biochemical parameters*

2.4*a Haemolymph preparation*: Five random samples from 5th instar larvae in their 6th day were selected for each treatment and one of their prolegs was cut. The haemolymph was collected in micro-tubes and 1 mg phenylthiourea added immediately to prevent melanization. The samples were centrifuged at 14,000 rpm for 10 min. The supernatant was removed and kept in – 20°C for analysis.

2.4*b Analysis*: Protein was measured by a total protein assay kit (Darmankave Co., Iran) with bovine serum albumin as the standard protein. Uric acid contents in the haemolymph were determined using uricase as described by Valovage and Brooks (1979). The concentration of urea was determined by measuring ammonia produced from urea, using a commercial urea assay kit (Chemenzyme Co., Iran). Glucose concentration of haemolymph was determined by the hexokinase method of Siegert (1987). The Richmond (1973) method was used for the total cholesterol assessment of haemolymph. This method is based on the hydrolysis of cholesterol ester by cholesterol oxidase, esterase and peroxidase enzymes. Triacylglycerol of haemolymph was measured utilizing Buclo and David's (1973) methods, which are based on the hydrolysis of haemolymph triacylglycerol by enzymes such as lipase, glycerokinase, glycerol-3-phosphate oxidase and proxidase. Finally, measurement of released glycerol was colorimetric spectrophotometry at 520 nm. Sodium and potassium were measured by flamephotometry using a lithium internal standard. Calcium was determined by the method of Baginski *et al* (1973) and inorganic phosphorus of larval haemolymph was measured by the method of Sakamoto and Horie (1979).

2.5 *Statistical calculations*

Collected data were subjected to statistical analysis of variance test for significant differences in the measured parameters of the normal (control) and treated groups. For all analysis of variance the Duncan's multiple range test in SAS software was used (SAS 1997).

3. Results

3.1 *Larval characteristics*

High-dose of vitamin B_3 in silkworm diet interrupted feeding and normal growth of the larva. High mortality of larvae occurs during the ecdysis, and disrupts normal molting. Larvae also show symptoms of nicotinamide hypervitaminosis such as immobility, dyspepsia, darkening of the skin, inability to excrete properly, exerting brownish fluid from anus and swelling of rectal muscles.

The larval weight in vitamin-treated larvae shows a considerable decrease (table 1). The decrease of larval weight is completely dependent on concentration. The maximum larval weight was observed in 1 g/l treatment (2·9 g) showed 48% weight decrease compared to the control (table 1). Larger doses showed even greater weight decreases. There is also a significant difference between the growth rate of control insects and the vitamin-treated ones. In table 1 complete dependency of DIW percent or larval growth rate to concentration was observed.

The increase of larval stage duration in the vitamin-treated larvae was very noticeable. Larvae treated with 1, 2 and 3 g/l concentrations of nicotinamide survived for 18, 26 and 31 days respectively and were still feeding after the control insects went into the pupal stage.

3.2 *Cocoon characteristics*

The maximum cocoon weight was recorded in the 1 g/l treatment (table 2). Only 7 larvae from 100 larvae could transform into pupae and their mean cocoon weight was 1·9 g, which showed more than 30% weight decrease vs controls. However in 3 g/l concentration no larvae were able to pupate. Consequently after the decrease of larval weight, the pupal weight was affected by different concentrations of this vitamin. One g/l concentration caused more than 30% weight decrease of pupa although this reduction was almost 44% in the 2 g/l concentration. The cocoon shell weight decreased from 0·74 g in the control to 0·49 g in the 1 g/l treatment.

3.3 *Biochemical characteristics of haemolymph*

Most of the biochemical macromolecules that were measured showed a significant reduction with nicotinamide treatment except for the uric acid and glucose. Glucose levels did not change with treatment whereas the uric acid increased from 0·31 μg/ml in the 1 g/l treatment to 0·75 μg/ml in the 3 g/l treatment (control = 0·34 μg/ml).

Four minerals including sodium, potassium, phosphorus and calcium were measured in the various treatments of nicotinamide (figure 1). Results show that the amount

Table 1. The effect of nicotinamide on the silkworm larval weight (g).

Nicotinamide concentration (g/l)	4th instar 4th day	5th Instar 1st day	5th Instar 3rd day	5th Instar 5th day	5th Instar 7th day	DIW %
1	$0{\cdot}62^b$	$0{\cdot}93^b$	$1{\cdot}69^c$	$2{\cdot}71^b$	$2{\cdot}91^b$	$30{\cdot}3^b$
2	$0{\cdot}65^b$	$0{\cdot}86^c$	$1{\cdot}33^d$	$1{\cdot}59^c$	$1{\cdot}62^c$	$10{\cdot}8^c$
3	$0{\cdot}66^b$	$0{\cdot}86^c$	$1{\cdot}31^e$	$1{\cdot}12^d$	$1{\cdot}26^d$	$5{\cdot}5^d$
C	$0{\cdot}98^a$	$0{\cdot}99^a$	$2{\cdot}57^b$	$4{\cdot}22^a$	$5{\cdot}62^a$	$77{\cdot}7^a$
DWC	$0{\cdot}99^a$	$0{\cdot}98^a$	$2{\cdot}63^a$	$4{\cdot}41^a$	$5{\cdot}58^a$	$76{\cdot}6^a$

There is no significant difference between the numbers that are shown with the same letter in columns.
C, Normal control; DWC, distilled water control; DIW, daily increased weight.

Table 2. The effects of nicotinamide on the cocoon parameters of silkworm.

Nicotinamide concentration (g/l)	No.	Single cocoon characters (g) Cocoon weight	Pupa weight	Shell weight
1	7	$1{\cdot}88^b$ (31·5)[†]	$1{\cdot}39^b$ (30·5)	$0{\cdot}49^c$ (34)
2	2	$1{\cdot}68^c$ (39)	$1{\cdot}12^c$ (44)	$0{\cdot}55^d$ (25·21)
3	0	–	–	–
C	95	$2{\cdot}75^a$	$2{\cdot}00^a$	$0{\cdot}74^a$
DWC	90	$2{\cdot}72^a$	$2{\cdot}04^a$	$0{\cdot}68^b$

[†]The percentage of reduction between control and treatments.
There is no significant difference between the numbers that are shown with the same letter in columns.
C, Normal control; DWC, distilled water control.

of phosphorus in all the concentrations decreased more than 50%. The 1·2 mg/ml of phosphorus level in the haemolymph of control larvae was reduced to 0·62 to 0·75 mg/ml with nicotinamide treatment. Calcium in the haemolymph also was significantly decreased. In the treated larvae calcium was between 0·53 to 0·80 mg/ml while in the control and distilled water treatment was 1·02 and 1·06 mg/ml respectively. Sodium and potassium level in the haemolymph with nicotinamide treatment did not demonstrate any significant changes.

4. Discussion

Niacin treatment caused great disruption of larval and cocoon characteristics. In addition, many biochemical compounds decreased in the haemolymph as a result of high concentrations of this vitamin.

Etebari (2002) showed that the high concentrations of nicotinamide (10, 20 and 30 g/l) could cause intensive mortality in the larval stage. From the first instar, the larvae in this group were treated with high doses. Molting was disrupted in each stage and many larvae died. Many of larvae were unable to continue molting and spent the rest of their lives in the same stage. Only 1·2% of larvae could reach to fifth instar. None of the larvae from the 20 and 30 g/l concentrations entered the 5th instar. Etebari (2002) reported the maximum larval weights in the concentrations of 5, 10, 20 and 30 g/l are 139·6 (5th instar), 100·7 (5th instar), 30·3 (4th instar) and 28·2 (4th instar) mg respectively.

In the current study, the maximum-recorded larval weight in 5th instar larvae was in 1 g/l treatment with 2·9 g. There have previously been reports showing hypervitaminosis effects in other insects. Tsiropoulos (1985) described that when *Dacus oleae* (Gmel.) are given high-doses of biotin and pyridoxine, there will be decrease in the survival of both sexes. It is also thought that the tolerance to hypervitaminosis is different in male and female because Tsiropoulos and Cavalloro (1983) demonstrated that there is a greater sensitivity of female adults of olive fly to the concentration enhancement of biotin, pyridoxine, inositol and dihydroascorbic acid. In addition to mortality, the oviposition ability is also decreased significantly. The per centage of hatched eggs was also decreased by high concentrations of calcium pantothenate and biotin.

In the silkworm, Saha and Khan (1996) described the extensive effects of multi-vitamin compounds as diet factors in growth interruption and the decrease of cocoon charac-

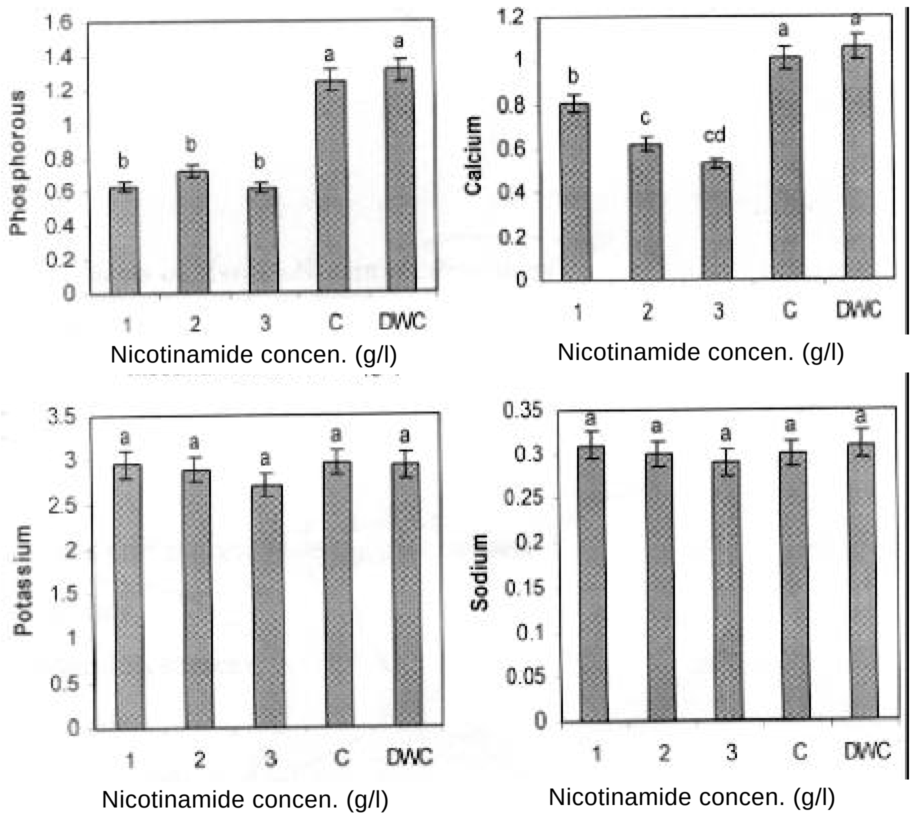

Figure 1. The effects of nicotinamide on some mineral ion in silkworm larvae (mg/ml). There is no significant difference between the numbers that are shown with the same letter in columns. C, Normal control; DWC, distilled water control.

Table 3. The effects of nicotinamide on biochemical macromolecules of 5th instar larval haemolymph.

Concentration (g/l)	Glucose (μg/ml)	Protein (mg/ml)	Urea (mg/ml)	Uric acid (μg/ml)	Triacylglycerol (mg/ml)	Cholesterol (μg/ml)
1	1·3[b]	0·45[b]	22·3[c]	0·3[c]	22·5[b]	1·9[b]
2	1·3[b]	0·38[c]	25·5[b]	0·5[b]	20·0[bc]	1·6[c]
3	1·2[b]	0·35[c]	18·0[d]	7·6[a]	18·5[c]	1·6[c]
C	1·8[a]	0·95[a]	30·8[a]	3·9[c]	45·0[a]	2·4[a]
DWC	2·0[a]	0·86[a]	26·5[b]	3·0[c]	47·5[a]	2·3[a]

There is no significant difference between the numbers that are shown with the same letter in columns.
C, Normal control; DWC, distilled water control.

teristics. Not only did the silkworm larvae fed high doses of vitamin C not show any increase in the cocoon characteristics, but a 5% decrease in cocoon weight was observed. Although this decrease in weight was observed in female cocoons, the males were able to tolerate the hypervitaminosis (Etebari *et al* 2004).

Etebari *et al* (2004) demonstrated that feeding a 3% concentration of ascorbic acid, decreases characteristics such as larval weight apparently due to hypervitaminosis. Excess amounts of vitamin in an insect's diet also have negative effects and decrease the feeding. Mcfarlane (1992) suggests that high levels of ascorbic acid can inhibit spermatogenesis in house crickets and decrease the viability of eggs produced. Kaur and Srivastava (1995) reported that extensive amounts of vitamin C on *Dacus cucurbitae* could interrupt its life cycle.

As a whole, treatment with niacin caused the decrease of all the biochemical compounds in haemolymph except uric acid. The enrichment of leaves with distilled water should not have considerable effect on biochemical compounds of the haemolymph. Since all the biochemical compounds of larval haemolymph decreased, finding a correlation between the above factors and the effect on biological parameters of the larva is not easy. Probably this decrease in measured values is due to the lack of feeding from leaves.

Starvation changes of the amount of some biochemical macromolecules in silkworm larval haemolymph (Etebari and Matindoost 2004). Our results show that all the measured compounds except uric acid and urea in fifth instar larvae had considerable reduction. Therefore, it is clear that nicotinamide has antifeedant effects on larvae and it interrupts the biology of the larvae, which consequently causes changes in biochemical compounds of haemolymph. Satake *et al* (2000) showed that the quality of the food taken by larvae and starvation would have considerable effects on the haemolymph glucose.

Besides the decrease of biological performance and causing biochemical changes of haemolymph, hypervitaminosis makes larval skin dark. In the tobacco hornworm *Manduca sexta*, dark pigmentation is a symptom of juvenile hormone deficiency. The black larval mutants in these insects are smaller as larvae, pupae and adults; lay fewer eggs and are more sensitive to desiccation (Safranek and Riddiford 1975).

Research show that there is a correlation between feeding activity and analogue structures of vitamins. In the analogues of niacin, carboxyl group (COOH–) of niacin in the *b* site of pyridine cycle could be replaced by $CONH_2$ or CHO without having any negative effects on feeding. But methyl, sulphon and acetyl groups can inactivate it. The compounds having carboxyl group in ***a*** and *g* sites do not show any activity. It has also been reported that the analogue of niacin, 4-acetyle pyridine when added to mulberry leaves interrupts the larval growth and causes niacin deficiency and acts as an anti metabolite. When added as a complex with niacin, antagonistic effects are expressed (Ito 1978), it tends to replace NAD in the reactions and consequently interrupts the metabolic pathway. Since nicotinamide co-enzymes act in reactions with very specific substrates this replacement inhibits the normal activities of insects (Jang 1986).

Horie and Ito (1965) showed the acquired level of niacin in silkworm is highly regulated to the most appropriate level of 33 μg/g of dry weight and that increasing this amount, reduced the larval weight. They have not explained the reasons and mechanism behind this reduction. Horie (1995) showed that with the increase of larval weight, the requirement of the silkworm for this vitamin was decreased. The essential amount of this vitamin for first and second instar larvae in the artificial diet is 0·008 g per 100 g of artificial diet. Chang *et al* (2000) in the study of the factors affecting the growth of *Ceratitis capitata*, found that it is essential for growth, in small quantities. Therefore, the silkworm's requirement (and also the need of most insects) for this vitamin is very limited and high doses of this vitamin can leave many negative effects.

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