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K. Lakshmi Devi
Sri Venkateswara University, Tirupati- 517502, Andhra Pradesh, India.

K. Yellamma
Sri Venkateswara University, Tirupati- 517502, Andhra Pradesh, India.

Correspondence:
K. Yellamma
Sri Venkateswara University, Tirupati- 517502, Andhra Pradesh, India.
Email: Yellamma55@gmail.com;
Tel: 9885248629

The Stimulatory Effect of Zinc, Pyridoxine and Methoprene on Cholinergic System in the Silkworm, *Bombyx mori* (L)

K. Lakshmi Devi, K. Yellamma

Abstract

The present study has been aimed at examining severe perturbations in the Cholinergic system in different tissues like Silk Gland, Hemolymph, Fat body and Muscle of silkworm larvae treated with Zinc, vitamin, Pyridoxine and hormone, Methoprene. The experimental worms were divided in to four groups and fed with mulberry leaves soaked in the selected compounds i.e. Zinc chloride, Pyridoxine, Methoprene and with Mixed dose (Zn+B6+H). The Control group of silkworm larvae was fed with normal mulberry leaves. All groups of silkworm larvae were fed four times in a day throughout the 5th instar larval period. Both Control and Experimental silkworm larvae were weighed daily and sacrificed on selected days viz. 1st, 3rd, 5th and 7th day. Cumulatively, the findings of the present study suggest that the mixed dose significantly elevated the levels of ACh and declined the AChE activity in all selected experimental groups except Control.

Keywords: Methoprene, Mulberry leaves, Perturbations, Pyridoxine, Zinc.

1. Introduction

Neurotransmitters are endogenous chemicals which transmit signals from a neuron to a target cell across a synapse. Neurotransmitters are packaged into synaptic vesicles clustered beneath the membrane on the presynaptic side of a synapse, and are released into the synaptic cleft, where they bind to receptors in the membrane on the postsynaptic side of the synapse. Release of neurotransmitters usually follows arrival of an action potential at the synapse, but may also follow graded electrical potentials.

In silkworms, all developmental stages are controlled over by the neurosecretory system which in turn is regulated by the Central Nervous System of all the larval stages, the 5th instar larval stage in the silkworm assumes lot of significance since it is in this stage the silk gland develops rapidly and all the biochemical constituents such as Glycogen, Trehalose, Proteins and Lipids reach their peak levels. The synthesis of these essential biochemical constituents depend upon a number of dietary factors. The cocoons spinning activity is an important phase in the silkworm which produces the cocoons, the final product of the animal. This activity which lasts for 5 to 6 days requires continuous function of the nervous system and the muscular system where the Cholinergic and Glutamatergic neurotransmitters play key roles. Since, the larval stage is the only feeding stage in silkworm development, intake of balanced diet is very essential for silk production. By supplementing the diet with trace elements, minerals, vitamins, Juvenile Hormones etc. the various functions of neuromuscular system, reproductive system etc., can be modulated effectively.

i. Acetylcholine (ACh)

ACh, known as chemical mediator of various types of activities in the nervous system, is synthesized by condensation of choline and acetyl CoA in a reaction catalyzed by choline acetyltransferase present in the cytosol of the neuron. It is synthesized, stored and released from cholinergic neurons. Such neurons also synthesize choline acetyltransferase (ChAT), the enzyme that catalysis the formation of ACh (reaction-1), and acetylcholinesterase (AChE), that catalysis the hydrolysis of ACh (reaction-2)[1].

$$Choline + Acetyl\ CoA \xrightarrow{Choline\ Acetyl\ Transferase\ (ChAT)} ACh + CoA \quad (1)$$

$$ACh + H_2O \xrightarrow{Acetylcholinesterase\ (AChE)} Choline + Acetic\ acid \quad (2)$$

The role of ACh as neurotransmitter at synaptic junctions is well established[2]. ACh is released from the nerve endings of cholinergic fibers to effect synaptic transmission. After liberation, the ACh binds to specific ACh receptors of post synaptic membrane, there by affecting the post synaptic cell or neuron. Later ACh dissociates spontaneously from ACh receptors and is immediately hydrolyzed by Acetyl cholinesterase[3] in to acetic acid and choline.

The cholinergic receptors in the nervous system can be divided in to two groups: nicotinic and muscarinic receptors, which mimic the effects of Ach[4]. Nicotinic receptors are directly gated ion channels that are usually considered as mediators of acetylcholine. While muscarinic receptors are the main type of cholinergic receptors in the central nervous system. Nicotinic actions are typically quick in onset and short-lasting, and are blocked by an excess of nicotine or by curare-like agents. By contrast, muscarinic actions tend to be slow in onset and are prolonged. They are stimulated by muscarine and acetylcholine, and blocked by atropine and related drug compounds. Nicotinic AChRs are inotropic receptors permeable to sodium, potassium and chloride ions. They are stimulated by nicotine and acetylcholine. They are of two main types, muscle type and neuronal type. The former can be selectively blocked by curare and the latter by hexamethonium[5].

For example, Acetylcholine and acetylcholine esterase activity in different races of *Antheraea mylitta* (D) are reported to be high in the active stages of life cycle like other insects[6]. Levels of acetylcholine and acetylcholine esterase are reported to be minimum and steady in the hibernating (diapausing) stage of insects and therefore the present study was designed to fill the gap of information regarding the *Antheraea mylitta* (D) diapause.

ii. Cholinesterases

Cholinesterases can be defined as a group of serine esters capable of hydrolyzing esters of choline at a high rate. Cholinesterases are widely distributed among animals both in invertebrates and vertebrates and present even in locations where the ACh is not neurotransmitter. This hydrolytic enzyme is widely distributed[7] particularly in excitable tissues, such as nerve, muscle and electrogenic tissues. Plasma acetylcholinesterase is found in pancreas and heart[8].

Cholinesterases are of two types. They are:

A) True cholinesterases (AChE) and

B) Pseudo cholinesterases (or) non-specific cholinesterases (BuChE).

iii. Acetylcholinesterase (AChE): AChE is one of the most extensively studied enzymes with respect to its mechanism of action. The active site of AChE comprises two subsites critical for its functioning. One of these has traditionally been termed the anionic subsite because it was hypothesized to consist of one or more negatively charged groups that electrostatistically interact with the positively charged quaternary nitrogen (N) of the substrate. The other region of active site is esteratic subsite, which possesses a serine residue that is responsible for driving the hydrolytic reaction[9].

AChE is found in synapses of both central and peripheral nervous systems, as well as neuromuscular junctions[10] and plays a major role in normal neuronal and neuromuscular transmission. Acetylcholinesterase is an enzyme that modulates the amount of neurotransmitter substance acetylcholine at nerve cell junction [11] and responsible for terminating the action of acetylcholine at synapses. AChE has a very high catalytic activity; each molecule of AChE degrades about 25000 molecules of acetylcholine per second. The choline produced by the action of AChE is recycled and transported through reuptake, back into nerve terminals where it is used to synthesize new acetylcholine molecules.

AChE is a glycoprotein made up of one or more subunits, each with a molecular weight of 70-80 KDa, depending on the species. The asymmetric form of AChE possesses a tail made up of three collagen filaments wound in a helical arrangement. Disulphide bonds (-S-S-) play a role in linking the subunits to each other and also to the tail. This form of AChE is found mainly at the neuromuscular junction, where it is secreted by muscle and nerve cells. AChE has also been hypothesized to play developmental roles in the nervous system and Col Q is also expressed in some AChE poor tissues[12].

AChE has also been found in several non-excitable tissues, most notably RBC and also liver, kidneys and placenta[13]. AChE has dual functions: on the postsynaptic side, it prevents the released ACh from acting longer than is necessary, and on presynaptic side, it helps to ensure an adequate supply of choline for ACh synthesis. Inhibitors of AChE directly act as cholinergic agonists by blocking the mechanism by which the neurotransmitter acetylcholine is removed from the synaptic cleft. It has been shown that inhibition of AChE leads to accumulation of ACh, cholinergic hyper activity, convulsions and status epilepticus[14].

In addition to AChE, a related enzyme pseudocholinesterase or butyrylcholinesterase (BuChE) occurs in the nerves and muscles of vertebrates. The sum of AChE and BuChE activities is usually termed cholinesterase activity[15]. BuChE are less specialized enzymes when compared with AChE. They slowly react with acetylcholine and also react with a wide range of esters. In view of the role of cholinergic system as a neurotransmitter in the brain, an attempt has been made in the present study to examine the neuro protective role of various nutrients on cholinergic system in different tissues of control and experimental silk worms.

iv. Enzyme structure and mechanism

AChE has a very high catalytic activity - each molecule of AChE degrades about 25000 molecules of acetylcholine (ACh) per second, approaching the limit allowed by diffusion of the substrate[16]. The active site of AChE comprises 2 subsites - the anionic site and the esteratic subsite. The structure and mechanism of action of AChE have been elucidated from the crystal structure of the enzyme[17]. The anionic subsite accommodates the positive quaternary amine of acetylcholine as well as other cationic substrates and inhibitors. The cationic substrates are not bound by a negatively-charged amino acid in the anionic site, but by interaction of 14 aromatic residues that line the gorge leading to the active site[18]. All 14 amino acids in the aromatic gorge are highly conserved across different species[19].

For a cholinergic neuron to receive another impulse, ACh must be released from the ACh receptor. This occurs only when the concentration of ACh in the synaptic cleft is very low. Inhibition of

AChE leads to accumulation of ACh in the synaptic cleft and results in impeded neurotransmission. Irreversible inhibitors of AChE may lead to muscular paralysis, convulsions, bronchial constriction, and death by asphyxiation. Organophosphates (OP), esters of phosphoric acid, are a class of irreversible AChE Cleavage of OP by AChE leaves a phosphoryl group in the esteratic site, which is slow to be hydrolyzed (on the order of days) and can become covalently bound. AChE is found in many types of conducting tissue: nerve and muscle, central and peripheral tissues, motor and sensory fibers, and cholinergic and non-cholinergic fibers.

2. Materials and Methods

Test species : Silkworm, *Bombyx mori* (Disease-free larvae from local grainages)
Mulberry : M_5 Variety
Larval Instar : 5th Instar
Test chemicals : 1. Zinc chloride (Fisher Inorganic & Aromatics Ltd,
2. Pyrol / Pyridoxine hydrochloride (vitamin B6) (FI & AL)
3. Methoprene Hormone (Seri-Agro market: Bangalore)
Duration of treatment : 7 Days
Dose Selected
Zinc chloride : 2 µg/ml
Pyridoxine hydrochloride : 2 µg/ml
Methoprene Hormone : 2 µg/ml
Tissues Selected : Silk Gland, Hemolymph, Fat body and Muscle

i. Test species:

The present investigation was carried out on the Pure Mysore x CSR_2 hybrid variety of the silkworm, *Bombyx mori*. Since the experiments required continuous maintenance of the test species, silkworms were reared in the laboratory itself in accordance with the procedure[20].

ii. Treatment of fifth instar larvae with Zinc, Pyridoxine and Methoprene:

Required concentration (2 µg/ml) of Zinc chloride, Pyridoxine and Methoprene solutions were prepared in distilled water as shown below.

iii. Preparation of standard stock solutions

For the preparation of standard stock solution 1, 1 gm of zinc chloride was dissolved in 100 ml of distilled water (1000 mg x 1000 µg /100 ml) which is equivalent to 10000 µg/ml. From this solution 1 ml was taken and added to 99 ml of distilled water (10000 µg /100 ml) which is equivalent to 100 µg/ml, known as standard stock solution 2. The same procedure was followed in the case of Pyridoxine, Methoprene and Mixed dose also.

For the preparation of 2 µg/ml concentration, 2 ml of standard stock solution 2 was added to 98 ml of distilled water. But in the preparation of mixed dose, the above prepared standard stock solution 2, each 2ml i.e. Zinc +Pyridoxine + Methoprene (2 ml+2 ml+2 ml) was added to 94 ml of distilled water. 100 ml of each of these concentrations were prepared as per the above table and 25-75 mulberry leaves were soaked in these solutions, dried at room temperature till the wetness is removed & were used to feed four groups of experiment larvae of the 5th instar stage for 7 days.

iv. Estimation of Acetylcholine and Acetylcholinesterase:

A) Acetylcholine: The larvae were sacrificed and the selected tissues were extracted for estimation. The tissues were weighed accurately on electronic balance. Homogenates of different tissues of larvae were prepared separately through the use of pre chilled mortar and pestle in glass distilled water 10 percent by weight and volume. Acetylcholine content of homogenate was determined through the method described by, a method of[21] as given by Augustinsson (1957). Different tissues of the silkworm like silk gland, hemolymph, fat body and muscle weighed accurately, transferred to test tubes and placed in a boiling water bath for 5 minutes to terminate the Acetyl cholinesterase enzyme activity and also to release the bound Acetylcholine (ACh). Then the tissues were homogenized in 1 ml of distilled water. To the homogenate, 1 ml of alkaline hydroxylamine hydrochloride was added followed by 1 ml of 50% hydrochloric acid solution. The contents were mixed thoroughly and centrifuged. To the supernatant, 0.5 ml of 0.37 M ferric chloride solution was added and the brown colour developed was read at 540 nm against a reagent blank (1 ml of distilled water and 0.5 ml of 0.37 M ferric chloride solution) in a spectrophotometer. The acetylcholine content was expressed as μ moles of ACh/g wet weight of tissue.

The 10 mL of homogenate was mixed in the 10 mL of boiling acidified Ringer solution of pH = 5. The mixture was kept at room temperature for half an hour and then subjected for centrifugation at 400 rpm. The supernatant was transferred to clean test tube and used as assay sample for acetylcholine content analysis.

B) Acetylcholinesterase (AChE): (E.C.: 3.1.1.7; Acetylcholine acetyl hydrolase): Acetylcholinesterase (AChE) activity was estimated by the method of[22] 10% homogenates of selected tissues of silkworm were prepared in 0.25 M ice-cold sucrose solution. The reaction was started with the addition of 100 μ liters of homogenate to the reaction mixture containing 3.0 ml of phosphate buffer (P^H 8.0), 20 μ moles of substrate, Acetylcholine iodide (0.075 M) and 100 μ moles of Dithiobis trinitrobenzene acid (DTNB 0.01 M). The developed colour was read at 412 nm in a spectrophotometer. The enzyme activity was expressed as μ moles of ACh hydrolyzed/mg protein/hour.

3. Statistical Analysis of Data

Values of the measured parameters were expressed as Mean±SEM. Repeated Measures of ANOVA was used to test the significance of difference among four different groups followed by *Dunnett's* Multiple Range Test (DMRT). Statistical analysis was performed by using Statistical Program of Social Sciences (SPSS) for windows (Version 19; SPSS Inc., Chicago, 1L, USA). The results were presented with the F-value and p-value. In all cases F-value was found to be significant with p-value less than 0.01**. This indicates that the effects of factors are significant.

4. Results

The results of the present study clearly indicate that the mixed doseinc + Pyridoxine + Methoprene has significantly altered the level of both Acetylcholine (ACh) content and Acetylcholinesterase (AChE) level in the cholinergic system of all selected tissues in control and all groups of experimental silk worm when compared with individual treatments with these compounds separately.

A) Acetylcholine (ACh) content: (Table 1 to 4 and Figs: 1 to 4)

i. Control silkworms

The acetylcholine content in the silk gland, hemolymph, fat body and muscle of the control silkworm was 2.052, 3.047, 1.022 and 4.030 µ moles of ACh/gm. wet weight of tissue, respectively and of all the four tissues, muscle has highest content while fat body had the lowest level when compared to other tissues as shown below:

Muscle	>	Hemolymph	>	Silk gland	>	Fat body
(4.030)		(3.047)		(2.052)		(1.022)

ii. Experimental silkworms Treated with 2 µg/ml Zinc chloride

On comparison with the control ones, ACh levels in all the experimental groups showed considerable changes in all selected tissues of silk worm. A significant elevation was noticed in zinc treated group at all time periods and the percentage of elevation was kept on increasing from day-1 to day-7. Maximum ACh content was noticed in muscle (5.050) followed by hemolymph (4.030), silk gland (3.122), and fat body (2.047).

Muscle > Hemolymph > Silk gland > Fat body

iii. Experimental silkworms Treated with Pyridoxine:

Similarly, in silk worm treated with vitamin B6, Pyridoxine ACh content showed a significant increase in all selected tissues of silk worm at selected time intervals. From day-1 to day-7. There was a gradual increase in the levels of ACh and the maximum ACh content was noticed in the muscle (5.372) followed by Hemolymph (4.375), silk gland (3.280) and fat body (2.357). However, the level of elevation in ACh content was almost same in silkworms treated with Zinc and Pyridoxine.

Muscle > Hemolymph > Silk gland > Fat body

iv. Experimental silkworms Treated with Methoprene:

On comparison with the E1 and E2 groups, the silk worms treated with methoprene showed a slightly higher level of elevation in ACh content from day-1 to day-7. A significant elevation was noticed in Methoprene treated group at all time periods and the percentage of elevation was kept on increasing from day-1 to day-7. Maximum ACh content was noticed in muscle (5.667) followed by Hemolymph (4.670), Silk gland (3.585) and fat body (2.667).

Muscle > Hemolymph > Silk gland > Fat body

v. Experimental silkworms Treated with Mixed dose:

In the case of mixed dose treated silkworms, the ACh content registered highest level of increase on 7th day with respect to the other experimental groups. Maximum elevation was recorded on 7th day in muscle (5.967) followed by Hemolymph (4.965), Silk gland (3.890) and fat body (2.972).

Muscle > Hemolymph > Silk gland > Fat body

One common observation made in the present experiment was, elevation in ACh content was significant and uniform in all selected tissues under different treatments, on all selected days during the 5th instar larval stage and the effect of Mixed dose(Zn+B6+H) was more pronounced when compared with the individual doses in the following order.

Muscle > Hemolymph > Silk gland > Fat body

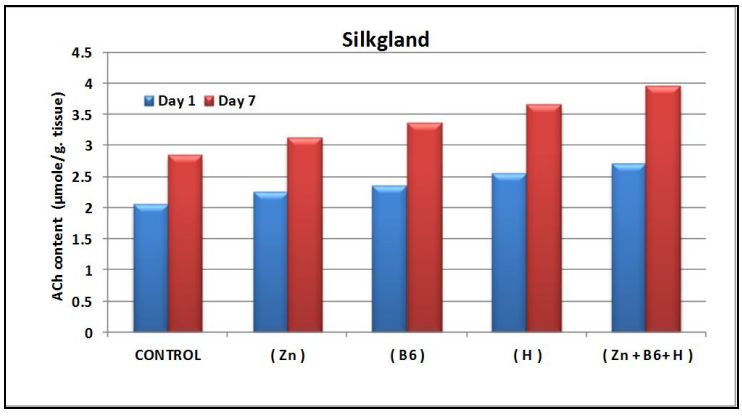


Fig 1: Changes in Acetylcholine content (µmoles of ACh/gm) in the Silk gland of Control and different Experimental groups of 5th instar silkworms

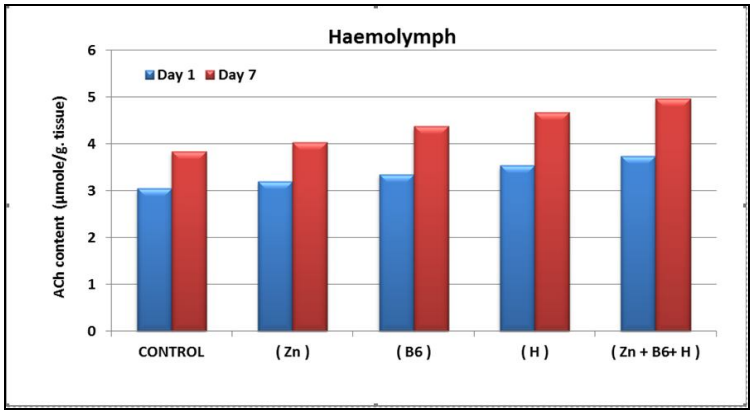


Fig 2: Changes in Acetylcholine content (µmoles of ACh/gm) in the Hemolymph of Control and different Experimental groups of 5th instar silkworms

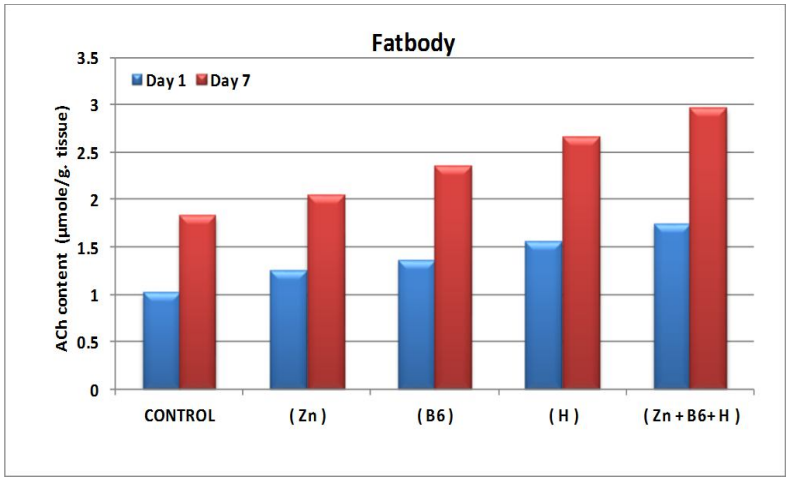


Fig 3: Changes in Acetylcholine content (µmoles of ACh/gm) in the Fat body of Control and different Experimental groups of 5th instar silkworms

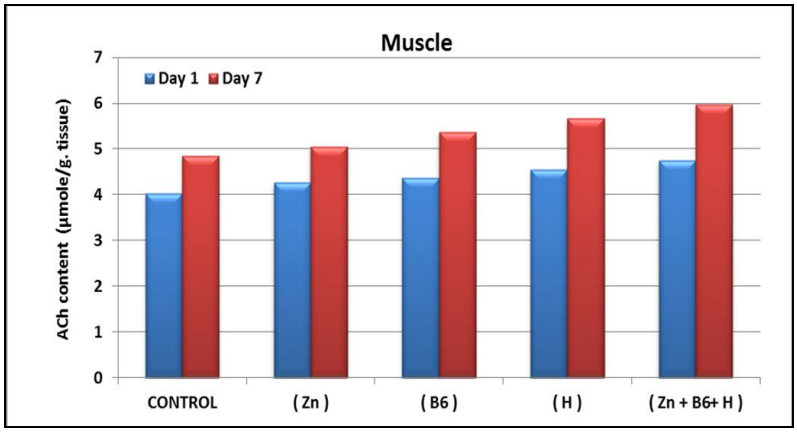


Fig 4: Changes in Acetylcholine content (µmoles of ACh/gm) in the Muscle of Control and different Experimental groups of 5th instar silkworms

Table 1: Changes in Acetylcholine content (µmole of ACh/gm) in the Silk gland of Control and Experimental groups of 5th instar Silkworms

Selected Days Of 5th Instar		CONTROL	E – I (Zn)	E – II (B6)	E – III (H)	E – IV (Zn + B6+ H)
DAY – 1	Mean	2.052	2.255	2.357	2.557	2.702
	Pc	-	(9.76)	(14.63)	(24.40)	(31.71)
	Sd	±0.03	±0.04	±0.04	±0.04	±0.04
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001
DAY – 3	Mean	2.335	2.597	2.650	2.842	2.915
	Pc	-	(11.60)	(13.73)	(20.26)	(25.32)
	Sd	±0.03	±0.02	±0.04	±0.03	±0.03
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001
DAY – 5	Mean	2.532	2.810	2.885	3.235	3.437
	Pc	-	(11.16)	(14.62)	(24.90)	(34.78)
	Sd	±0.02	±0.1	±0.02	±0.03	±0.03
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001
DAY – 7	Mean	2.840	3.122	3.362	3.652	3.955
	Pc	-	9.86	(15.80)	(30.28)	(37.32)
	Sd	±0.02	±0.1	±0.03	±0.03	±0.1
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001

Values are Mean±SEM of four observations each from tissues pooled from 4 silkworms
Values in parentheses are percent change from control
Values are significantly different from control at p < 0.01

Table 2: Changes in Acetylcholine content (µmole of ACh/gm.) in the Hemolymph of Control and Experimental groups of 5th instar Silkworms

Selected Days Of 5th instar		Control	E – I (Zn)	E – II (B6)	E – III (H)	E – IV (Zn + B6+ H)
Day – 1	Mean	3.047	3.197	3.347	3.537	3.745
	Pc	-	(11.27)	(9.87)	(16.12)	(23.03)
	Sd	± 0.02	±0.01	±0.02	±0.01	±0.01
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001
Day – 3	Mean	3.350	3.535	3.617	3.827	3.967
	Pc	-	(5.37)	(7.76)	(14.03)	(18.21)
	Sd	±0.02	±0.01	±0.02	±0.02	±0.01
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001
Day – 5	Mean	3.565	3.732	3.940	4.230	4.440
	Pc	-	(4.78)	(10.67)	(18.82)	(23.03)
	Sd	±0.01	±0.01	±0.03	±0.01	±0.03
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001
Day – 7	Mean	3.840	4.030	4.375	4.670	4.965
	Pc	-	(4.95)	(13.80)	(21.61)	(29.43)
	Sd	±0.01	±0.01	±0.02	±0.01	±0.01
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001

Values are Mean±SEM of four observations each from tissues pooled from 4 silkworms
Values in parentheses are percent change from control
Values are significantly different from control at p < 0.01

Table 3: Changes in Acetylcholine content ((µmole of ACh/gm.) in the Fat body of Control and Experimental groups of 5th instar Silkworms

Selected Days Of 5thinstar		CONTROL	E – I (Zn)	E – II (B6)	E – III (H)	E – IV (Zn + B6+ H)
DAY – 1	Mean	1.022	1.260	1.365	1.555	1.752
	Pc	-	(23.53)	(33.33)	(51.96)	(71.57)
	Sd	±0.01	±1.0	±0.01	±0.1	±0.01
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001
DAY – 3	Mean	1.360	1.547	1.637	1.837	1.932
	Pc	-	(13.24)	(19.85)	(34.56)	(41.91)
	Sd	±0.01	±0.02	±0.01	±0.01	±0.01
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001
DAY – 5	Mean	1.552	1.745	1.932	2.235	2.453
	Pc	-	(12.90)	(24.52)	(44.52)	(58.06)
	Sd	±0.01	±0.01	±0.02	±0.03	±0.01
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001
DAY – 7	Mean	1.835	2.047	2.357	2.667	2.972
	Pc	-	(12.02)	(28.96)	(45.90)	(62.30)
	Sd	±0.02	±0.01	±0.03	±0.01	±0.01
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001

Values are Mean±SEM of four observations each from tissues pooled from 4 silkworms
Values in parentheses are percent change from control
Values are significantly different from control at p < 0.01

Table 4: Changes in Acetylcholine content (µmole of ACh/gm.) in the Muscle of Control and Experimental groups of 5th instar Silkworms

Selected Days of 5th Instar		Control	E – I (Zn)	E – II (B6)	E – III (H)	E – IV (Zn + B6+ H)
DAY – 1	Mean	4.030	4.260	4.362	4.557	4.757
	Pc	-	(5.71)	(8.20)	(13.15)	(18.11)
	Sd	±0.01	±0.01	±0.01	±0.01	±0.01
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001
DAY – 3	Mean	4.360	4.552	4.655	4.855	4.940
	Pc	-	(4.36)	(6.88)	(11.47)	(13.30)
	Sd	±0.01	±0.02	±0.02	±0.02	±0.01
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001
DAY – 5	Mean	4.555	4.757	4.957	5.235	5.467
	Pc	-	(4.40)	(8.77)	(14.91)	(19.96)
	Sd	±0.01	±0.01	±0.01	±0.03	±0.01
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001
DAY – 7	Mean	4.840	5.050	5.372	5.667	5.967
	Pc	-	(4.34)	(10.95)	(17.15)	(23.35)
	Sd	±0.01	±0.01	±0.02	±0.01	±0.01
	T	-	P < 0.001	P < 0.001	P < 0.001	P < 0.001

Values are Mean±SEM of four observations each from tissues pooled from 4 silkworms
Values in parentheses are percent change from control
Values are significantly different from control at p < 0.01

B) Acetylcholinesterase (AChE) activity: (Table 5 to 8 and Figs: 5 to 8)

The specific activity of AChE in control silk worm was recorded highest in silk gland (14.72) followed by at body (13.94), muscle (11.95) and Hemolymph (6.23).

Sg	>	Fb	>	Mc	>	Hl
(14.72)		(13.94)		(11.95)		(6.23)

With respect to the control group, all the experimental groups showed significant changes in different tissues of silk worm. Contrary to the ACh content, the AChE activity had declined significantly in all the selected tissues of v-instar silk worm larvae treated with Zinc at selected time intervals. The percent of inhibition was gradually increased from day-1 to day-7 and the maximum inhibition was noticed in Hemolymph (-33.38%) followed by Silk gland (-26.13%), Muscle (-23.55) and Fat body (-16.71%).

Hl	>	Sg	>	Mc	>	Fb
(-33.38%)		(-26.13%)		(-23.55%)		(-16.71%)

When compared to control group, the AChE activity of Pyridoxine treated silk worm larvae exhibited more inhibition which gradually increased from day-1 to day-7. The percent of inhibition was gradually increased and the maximum percent of inhibition was recorded in Hemolymph (-35.47%) followed by Silk gland (-32.53%), Muscle (-27.82%) and Fat body (-25.87%).

Hl	>	Sg	>	Mc	>	Fb
(-35.47%)		(-32.53%)		(-27.82%)		(-25.87%)

In parallel to the above two groups, AChE activity in Methoprene treated silk worm larvae also recorded significant Inhibition gradually from day-1 to day-7 and maximum inhibition was recorded in Hemolymph (-44.62%) followed by Silk gland (-37.55%), Muscle (-30.46%) and Fat body (-28.41%).

Hl	>	Sg	>	Mc	>	Fb
(-44.62%)		(-37.55%)		(-30.46%)		(-28.41%)

Similarly, AChE activity has recorded highest inhibition in silkworms which received the mixed dose (Zn+B6+H) on 7th day with respect to the other experimental groups. The percent of inhibition was gradually increased from 1st day to 7th day and maximum percent change was noticed in Hemolymph (-48.47%) followed by Silk gland (-38.57%), Muscle (-34.82) and Fat body (-31.13%).

Hl	>	Sg	>	Mc	>	Fb
(-48.47%)		(-38.57%)		(-34.82%)		(-31.13%)

From the above results, it was observed that the AChE activity had declined significantly in the silk gland, Hemolymph, fat body and the muscle of the silkworms on treatment with selected doses of Zinc, Pyridoxine, Methoprene on all selected days during the 5th instar larval stage and the effect of Mixed dose (Zn+B6+H) was more pronounced.

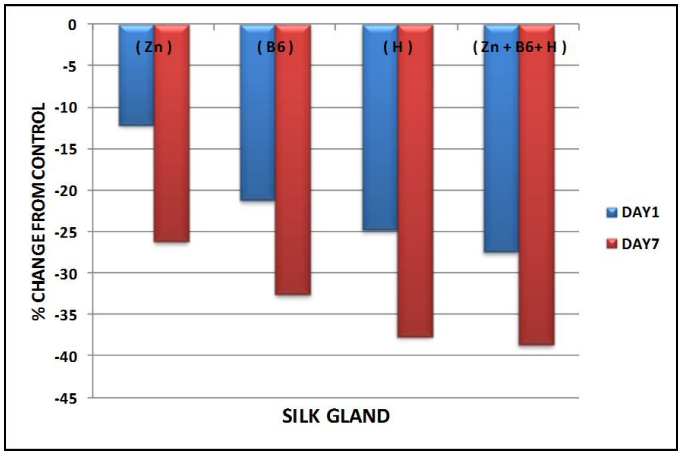


Fig 5: Percent changes in the activity of Acetylcholinesterase in the Silk gland of control and different Experimental groups of 5th instar silkworms

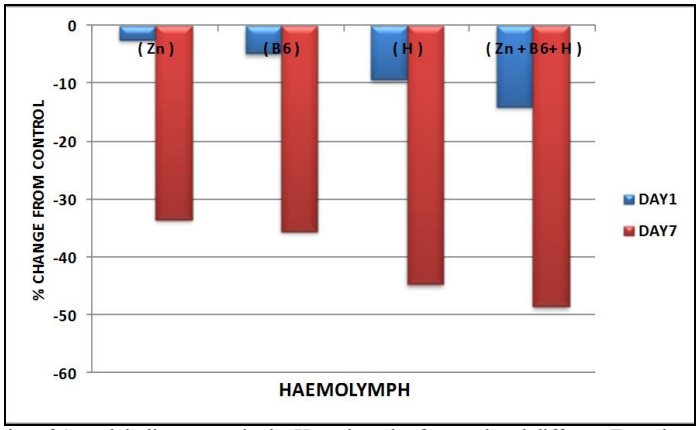
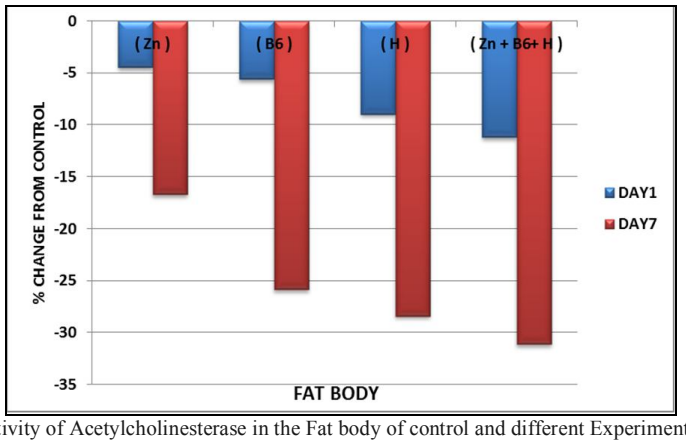


Fig 6: Percent changes in the activity of Acetylcholinesterase in the Hemolymph of control and different Experimental groups of 5th instar silkworms



Figc7: Percent changes in the activity of Acetylcholinesterase in the Fat body of control and different Experimental groups of 5th instar silkworms

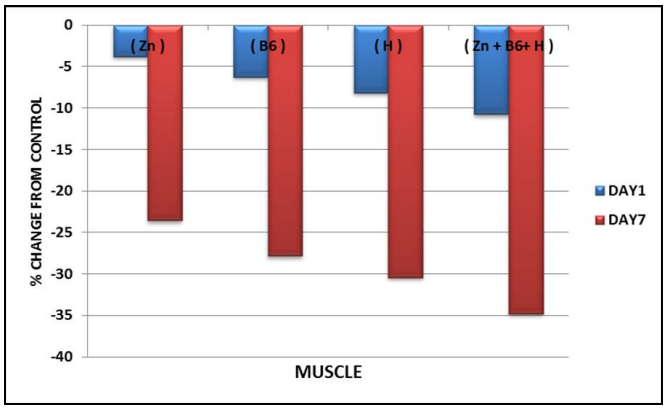


Fig 8: Percent changes in the activity of Acetylcholinesterase in the Muscle of control and different Experimental groups of 5th instar silkworms

Table 5: Changes in Acetylcholinesterase activity (µmoles of ACh hydrolysed/mg protein/h) in the silk gland of Control and Experimental groups of5th instar silkworms

Selected Days of 5th Instar		Control	E – I (Zn)	E – II (B6)	E – III (H)	E – IV (Zn + B6+ H)
DAY – 1	Mean	12.46	10.96	9.82	9.38	9.05
	Pc	-	(-12.04)	(-21.18)	(-24.72)	(-27.37)
	Sd	±0.01	±0.01	±0.02	±0.01	±0.02
DAY – 3	Mean	12.75	10.65	9.63	8.94	8.74
	Pc	-	(-16.47)	(-24.47)	(-29.88)	(-31.61)
	Sd	±0.02	±0.03	±0.01	±0.02	±0.02
DAY – 5	Mean	13.47	10.43	9.47	8.74	8.64
	Pc	-	(-22.57)	(-29.70)	(-35.15)	(-35.86)
	Sd	±0.01	±0.01	±0.02	±0.01	±0.01
DAY – 7	Mean	13.74	10.15	9.270	8.58	8.44
	Pc	-	(-26.13)	(-32.53)	(-37.55)	(-38.57)
	Sd	±0.01	±0.02	±0.01	±0.01	±0.01

Values are Mean±SEM of four observations each from tissues pooled from 4 silkworms
Values are Mean±SEM of four observations each from tissues pooled from 4 silkworms
Values in parentheses are percent change from control
Values are significantly different from control at $p < 0.01$

Table 6: Changes in Acetylcholinesterase activity (µmoles of ACh hydrolysed/mg protein/h) in the Hemolymph of Control and Experimental groups of 5th instar silkworms

Selected Days of 5thI Instar		Control	E – I (Zn)	E – II (B6)	E – III (H)	E – IV (Zn + B6+ H)
DAY – 1	MEAN	4.64	4.53	4.42	4.21	4.02
	PC	-	(-2.37)	(-4.74)	(-9.27)	(-13.36)
	SD	±0.01	±0.02	±0.02	±0.01	±0.01
DAY – 3	MEAN	4.83	4.42	4.21	4.08	3.70
	PC	-	(-12.63)	(-12.84)	(-15.53)	(-23.40)
	SD	±0.03	±0.02	±0.01	±0.01	±0.01
DAY – 5	MEAN	5.81	4.22	4.13	3.70	3.41
	PC	-	(-27.37)	(-28.92)	(-36.32)	(-41.31)
	SD	±0.01	±0.01	±0.01	±0.01	±0.01
DAY – 7	MEAN	6.23	4.15	4.02	3.45	3.21
	PC	-	(-33.38)	(-35.47)	(-44.62)	(-48.47)
	SD	±0.02	±0.02	±0.01	±0.01	±0.03

Values are Mean±SEM of four observations each from tissues pooled from 4 silkworms
Values in parentheses are percent change from control
Values are significantly different from control at $p < 0.01$

Table 7: Changes in Acetylcholinesterase activity (µmoles of ACh hydrolyzed/mg protein/h) in the Fat body of Control and Experimental groups of 5th instar silkworms.

Selected Days of 5th Instar		Control	E – I (Zn)	E – II (B6)	E – III (H)	E – IV (Zn + B6+ H)
DAY – 1	Mean	9.82	9.38	9.27	8.94	8.74
	Pc	-	(-4.48)	(-5.60)	(-8.96)	(-11.20)
	Sd	±0.01	±0.01	±0.01	±0.02	±0.02
DAY – 3	Mean	10.43	9.27	9.05	8.74	8.64
	Pc	-	(-11.12)	(-13.23)	(-16.20)	(-17.16)
	Sd	±0.01	±0.01	±0.02	±0.02	±0.01
DAY – 5	Mean	10.96	9.42	8.94	8.58	8.26
	Pc	-	(-14.05)	(-18.43)	(-21.71)	(-24.63)
	Sd	±0.01	±0.01	±0.02	±0.01	±0.01
DAY – 7	Mean	11.79	9.82	8.74	8.44	8.12
	Pc	-	(-16.71)	(-25.87)	(-28.41)	(-31.13)
	Sd	±0.01	±0.01	±0.01	±0.01	±0.02

Values are Mean±SEM of four observations each from tissues pooled from 4 silkworms
Values in parentheses are percent change from control
Values are significantly different from control at $p < 0.01$

Table 8: Changes in Acetylcholinesterase activity (µmoles of ACh hydrolysed/mg protein/h) in the Muscle of Control and Experimental groups of 5th instar silkworms

Selected Days of 5th Instar		Control	E – I (Zn)	E – II (B6)	E – III (H)	E – IV (Zn + B6+ H)
DAY – 1	Mean	8.44	8.12	7.93	7.75	7.53
	Pc	-	(-3.79)	(-6.28)	(-8.17)	(-10.78)
	Sd	± 0.01	±0.02	±0.02	±0.02	±0.03
DAY – 3	Mean	8.74	7.85	7.73	7.53	7.26
	Pc	-	(-10.18)	(-11.56)	(-13.84)	(-16.93)
	Sd	±0.01	±0.02	±0.02	±0.02	±0.02
DAY – 5	Mean	9.47	7.75	7.46	7.26	6.73
	Pc	-	(-18.16)	(-21.22)	(-23.34)	(-28.93)
	Sd	±0.02	±0.02	±0.02	±0.02	±0.02
DAY – 7	Mean	9.85	7.53	7.11	6.85	6.42
	P c	-	(-23.55)	(-27.82)	(-30.46)	(-34.82)
	Sd	±0.02	±0.03	±0.01	±0.02	±0.01

Values are Mean±SEM of four observations each from tissues pooled from 4 silkworms
Values in parentheses are percent change from control
Values are significantly different from control at $p < 0.01$

5. Discussion

The present findings demonstrated severe perturbations in the cholinergic system in different tissues of silkworm treated with Zinc, Pyridoxine, Methoprene and a combination of Zn+B6+H. From these results, it was obvious that oral administration of all the selected experimental nutrients and JH significantly elevated the levels of ACh and declined the AChE activity in all selected experimental groups except control. Acetylcholine, the primary transmitter of cholinergic system is one of the low molecular weight transmitter substances which are not an amino acid or derivative of amino acid[23]. Acetylcholine has functions both in the Central Nervous System (CNS) and in Peripheral Nervous System (PNS) as a neuromodulator. The role of ACh as neurotransmitter at synaptic junctions is well established long ago[2].

The present study demonstrated the changes that occur in the acetylcholine content in different tissues of silk worm control and experimental groups of V instar larvae of silk worm. The results clearly indicate that oral administration of Zinc exerted an elevation in the acetylcholine activity indicating that, Zinc has potential effect on the release of acetylcholine which can activate non-specific cation conductances to directly excite neurons[24]. Similarly, from the results of present study, it was obvious that oral administration of Pyridoxine and Methoprene significantly elevated the acetylcholine activity indicating that Pyridoxine and Methoprene have potential effect to cause a slow depolarization by blocking a tonically-active K+ current, which increases neuronal excitability. From the above results, it was obvious that oral administration of Mixed dose exerted more significant elevation in the acetylcholine activity indicating that, Mixed dose has more potential effect on the release of acetylcholine which can activate non-specific cation conductances to directly excite neurons.

Acetylcholine has other effects on neurons. One effect is to cause a slow depolarization by blocking a tonically-active K+ current, which increases neuronal excitability. Alternatively, acetylcholine can activate non-specific cation conductances to directly excite neurons[24]. Another effect upon postsynaptic M4-muscarinic ACh receptors is to open inward-rectifier potassium ion channel (Kir) and cause inhibition[25]. The influence of acetylcholine on specific neuron types can be dependent upon the duration of cholinergic stimulation. For instance, transient exposure to acetylcholine (up to several seconds) can inhibit cortical pyramidal neurons via M1 type muscarinic receptors that are linked to Gq-type G-protein alpha subunits. This suggests that proteins involved in controlling such processes would be regulated directly by Zinc[26]. In this way, metallothionein (MT), an extensively studied protein modulated by Zinc levels, helps to regulate the intracellular levels of free Zinc through intracellular binding. M1 receptor activation can induce calcium-release from intracellular stores, which then activates a calcium-activated potassium conductance which inhibits pyramidal neuron firing[27]. On the other hand, tonic M1 receptor activation is strongly excitatory. Thus, ACh acting at one type of receptor can have multiple effects on the same postsynaptic neuron, depending on the duration of receptor activation. Recent experiments on animal's behavior have demonstrated that cortical neurons indeed

experience both transient and persistent changes in local acetylcholine levels during cue-detection behaviors[28].

In general, the present investigation on cholinergic system in different regions of silk worm following the oral administration of Zinc, Pyridoxine, Methoprene and Mixed dose treated group have shown the neuro protective effect on cholinergic system by increasing the levels of ACh content and by inhibiting the AChE activity in all the experimental groups when compared to control. The Principal role of acetylcholinesterase (AChE) is the termination of nerve impulse transmission at the cholinergic synopses by rapid hydrolysis of acetylcholine (ACh). In summary, in this study the delineated cholinergic and non-cholinergic function of two AChE genes (Ace1 and Ace2) in *B. mori* was observed. AChE1 is an essential enzyme involved in cholinergic neurotransmission. In contrast, AChE2 plays important but non-cholinergic roles in insect growth, female reproduction and embryo development.

Two Ace genes reportedly display significant differences in tissue-specific expressions and molecular properties in the German cockroach (*Blattella germanica*)[29] the cat flea (*Ctenocephalides felis*)[30], and *T. castaneum*[31]. Functional differences of the two genes have not been adequately analyzed, although, based on selective and irreversible inhibition studies of aphid AChEs, it has been suggested that AChE2 does not contribute significantly to the overall AChE activity in aphids[32].

From the results of the present study, it was obvious that the changes that occur in the acetylcholinesterase activity in different tissues of experimental groups of V-instar larvae of silk worm were declined from day 1 to day 7. The results clearly indicate that oral administration of Pyridoxine and Methoprene also inhibited acetylcholinesterase activity indicating that, Pyridoxine and Methoprene have altered AchE. The reduced sensitivity of AChE to active inhibitors appears to have resulted from the reduced affinity of the enzyme to the inhibitor molecule rather than from the lowered rate constant for acylation[33]. The inhibition was more with Zinc, Pyridoxine, Methoprene and mixed dose treated groups except in control.

In view of the cholinergic and non-cholinergic functions of two AChEs in insects, it is suggested that any insect-specific anticholinesterase insecticides designed for insect pest control should target the AChE encoded by Ace1 rather than Ace2 unless the insect species possesses only Ace2. This notion is promoted by our findings that AChE1 in T. castaneum and many other insect species possesses a cysteine residue at the opening of the AChE active site but this cysteine residue is absent in fish and mammalian AChEs and insect AChE2[31]. This insect AChE1-specific cysteine residue allows design of new chemicals that irreversibly inhibit AChE1 for insect pest control by conjugation of the chemicals to the cysteine residue[32]. Indeed, several recent studies have demonstrated promising potencies of some synthetic chemicals in irreversibly inhibiting the total AChE activity of insects including *S. graminum* and *A. gambiae* but virtually no or limited inhibition to AChE from humans[32]. Such chemicals could potentially lead to the development of novel and environmentally-safe insecticides that are toxic to most insect species possessing Ace1 that is now known to be responsible for cholinergic neurotransmission[34].

In case of mixed dose treated silkworms, the acetylcholinesterase activity was more declined when compared to other experimental groups. The inhibition was maximum on 7th day when compared to other selected days. It means that the inhibition of altered AChE could be analyzed in vitro through the conventional biochemical experiments with suitable AChE inhibitors. Indeed, several recent studies have demonstrated promising potencies of some synthetic chemicals in irreversibly inhibiting the total AChE activity of insects including *S. graminum* and *A. gambiae* but virtually no or limited inhibition to AchE[32].

The AChE levels are inhibited in all the selected experimental groups viz., Zinc, Pyridoxine, Methoprene as well as in the Mixed dose treated group from day-1 to day-7. The present study demonstrates the changes that occur in the acetylcholinesterase activity in different tissues of silk worm control and experimental groups of V-instar larvae of silk worm. The results clearly indicate that oral administration of Zinc, Pyridoxine, Methoprene and Mixed dose inhibited the elevation in the acetylcholinesterase activity indicating that, they have altered AChE; the reduced sensitivity of AChE to active inhibitors appears to have resulted from the reduced affinity of the enzyme to the inhibitor molecule rather than from the lowered rate constant for acylation.

The present study substantiates that the reduced sensitivity of AChE to active inhibitors can be from the reduced affinity of the enzyme to the inhibitor molecule rather than from the lowered rate constant for acylation in resistant strains[33]. It means that the inhibition of altered AChE could be analyzed *in vitro* through the conventional biochemical experiments with suitable AChE inhibitors. In this study, the AChE activity was inhibited in all selected experimental groups is chosen as an AChEs inhibitor, and the sensitivity of other AChE inhibitors remains to be further studied.

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