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RESEARCH REPORT

Role of glutamate transporter EAAT2 in survival of silk worm *Bombyx mori* at higher dosages of Azaserine, an analogue of glutamine

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Abstract

Organisms are subjected to a variety of stress and physiological impact in the event of drug administration. Drug extrusion plays a critical role in the survival of organisms. The present study elucidate the role of glutamate transporter, EAAT2 in survival of model organism silkworm injected with azaserine, a glutamine analogue and offer a possible explanation for a contrasting observation of greater survival at higher dosage and lesser survival in lower dosage. Our results have shown a dosage dependent inhibition of gamma glutamyl transferase (GGT) activity, differential expression of EAAT2 transporter along with concomitant changes in residual concentration of azaserine, glutamate and cysteine that undermine cysteine transport. The current results also suggest that survival is dependent on EAAT2 transporter in cysteine-glutamate/azaserine transport and rejuvenation of ATP formation as observed in higher but not in lower dosage received worms.

Key Words: Azaserine, glutamate, EAAT2, GGT, GSH, ATP, RNAi, *Bombyx mori*

Introduction

Amino acid transporters play a crucial role in the metabolic dynamics in living organisms and these transporters primarily facilitate in cell signaling, that leads to either pro or anti apoptotic pathways**.** Amino acid L-Cystine is relevant in biological systems in wide array of processes and is a part of the antioxidant tripeptide molecule glutathione (GSH), which is composed of amino acids, glutamate, cysteine and glycine. Cystine is transported by several amino acid transporters like excitatory amino acid transporters EAAT1 and EAAT2, System ASC in invertebrates and system X_c in vertebrates (Lewerenz *et al.*, 2013). EAAT1 and EAAT2 co-transport cysteine with ($3Na^+/1H^+/AA$), as symporter with glutamate that has higher affinity to glutamate and as an antiporter with $1K^+$ (Chen and Swanson, 2003). System ASC is a Na^+-dependent antiporter that transports cysteine directly to exterior of the cell (Christensen, 1990). Once inside the cell, cystine is reduced to cysteine by the GSH or thioredoxin reductase (Mandal *et al.*, 2010) and incorporated into GSH that protects the cells against oxidative stress (OS) under various stressors, while the catabolism of GSH involves externalization of cysteine through gamma glutamyl transferases (GGT/γ-GT) (Dringen, 2000; Hanigan, 2014). Therefore a critical role is played by cystine/cysteine in maintaining cellular homeostasis, redox status and GSH metabolism under OS (Bannai *et al.*, 1989). During an OS there may be elevated externalization of glutamate that alters the status and can competitively inhibit cystine transport into the cells (Lewerenz *et al.*, 2013). The enzyme GGT is a glycoprotein located on the outer surface of the cell membrane, critical to cellular detoxification and amino acid transport (Keillor *et al.,* 2005). GGT also uses glutathione as an acyl donor substrate and transfers its glutamyl moiety to the acceptor substrate such as amino acid with higher affinity to L-cysteine and L-glycine and release cysteinyl glycine across the cell membrane (Allison, 1981). GGT has primary role in GSH catabolism and aids cystine/cysteine balance to maintain redox state. Cellular ATP is a biological source of energy and needed adequately during any form of stress, not only to counter stress and survive but also for normal cellular homeostasis and functioning. Drug azaserine is a glutamine analogue and is a competitive inhibitor for glutamine (Moore and Le page, 1957) and it can interact with cellular biomolecules thereby causing free radical generation and impairment in ATP generation (Revathi *et al.*, 2012). It also inhibits GGT irreversibly by binding covalently to the active site of

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Table 1 Sequence of the primers for mRNA of EAAT2 gene and tubulin for real time PCR

Genes Primer sequence	Product size	Accession number
SWTUB-FP: TCGTCGAGCCCTACAACTCT SWTUB-RP: ACTCGGTGAGGTCCACATTC	222 bp	NM_001043419.1
Bm-GluTran-qFP: AGATCACAGCATTCTGGATAGCTTGCTG Bm-GluTran-qRP: TGTGCCAGATCTGTAAGACACGACTCGT	187 bp	NM_001253896.1

the enzyme (Gardell and Tate, 1981) and also a non-competitive inhibitor in low K_m system for glutamine but has no effect on high K_m system for glutamine uptake (Hsu, 1980). The present work is based on a contrasting results obtained on azaserine treated silk worm larvae with higher survival rate that received higher dosage as compared to lower dosage. This prompted us to investigate the possible externalization of azaserine at higher dosages for subsequent higher rate of survival. Transport of azaserine/cysteine across membrane has been critically examined in erythrocytes (Yeldiz *et al*., 2009). We presume that azaserine-cystine transporter may be crucial and may pave way for azaserine into the cells/tissue because of its structural similarity to glutamine. Here we experimentally demonstrated the impact of varied dosages of azaserine on inhibition of cystine-glutamate transporter EAAT2. The aim of the present study includes the expression of the gene EAAT 2 in silk worm *Bombyx mori* through qPCR. RNAi studies were attempted in silencing the expression of the gene EAAT2 in order to authenticate the presence and functionality of the gene in *B. mori*. The other studies include the impact of azaserine residuality on GGT, GSH and ATP levels that plays a crucial role in the survival of injected larvae.

Materials and methods

Experimental animals

Healthy larvae of IV instar silk worm *Bombyx mori* were procured from licensed private rearing houses and were maintained until they attained V instar in laboratory conditions at 23 °C, humidity 75% and 12 L/D cycle, rearing of larvae was according to improved method of Krishnaswami (1978). 45 ± 5 larvae were injected with each concentration of azaserine. All the experiments were repeated for minimum six times (n=6).

Chemicals

All chemicals viz Azaserine, Cystine, Glutamate, Glutathione, 5-5'dithio (bis) nitrobenzoic acid (DTNB), γ- glutamyl-p-nitroanilide (GPNA), glycyl-glycine, creatine phosphate were obtained from Sigma Aldrich chemicals (St Louis, USA). RNA isolation kit, ds RNA, dNTP's, cDNA synthesis kit were obtained from Chromous Biotech (India). All other reagents were of HPLC grade and were procured from Himedia (India).

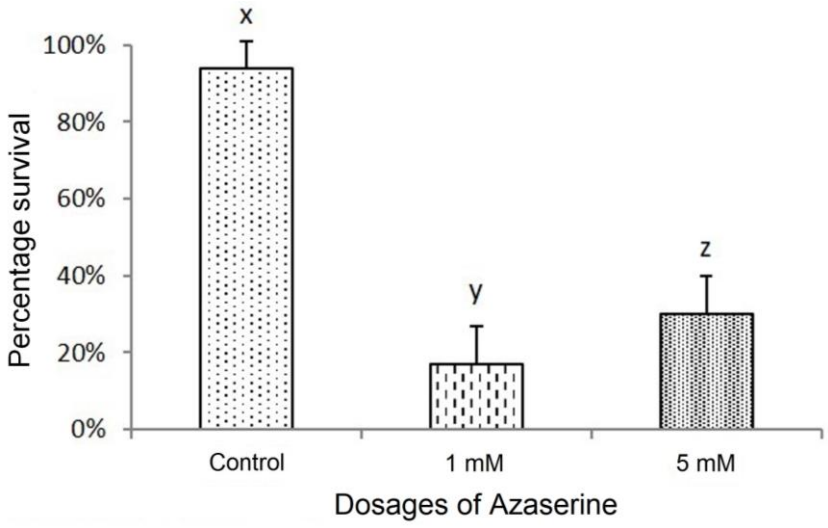

Fig. 1 Percentage of survival in larvae of *B. mori* injected with dosages of azaserine. Significance is represented as X, Y, Z. †Significance was calculated by MANOVA and post-hoc test was done using SPSS11.5 and significance was considered at $P < 0.05$

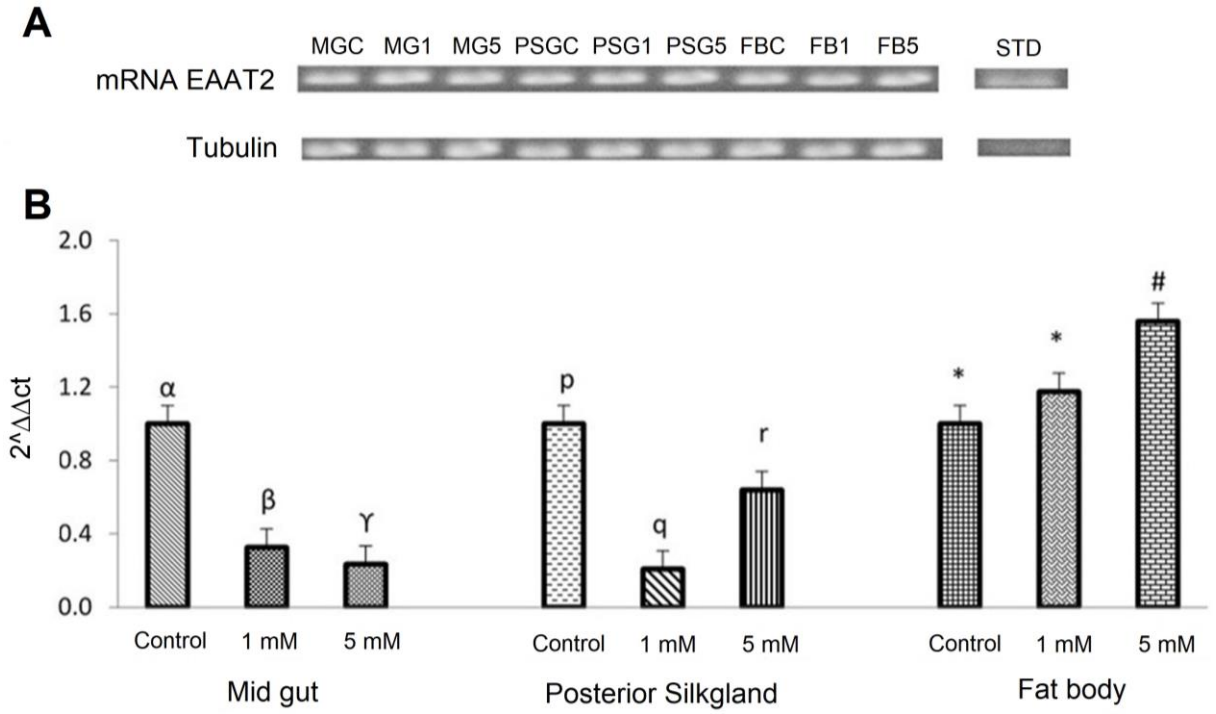

Fig. 2 A) representative agarose gel photograph from control, 1 and 5 mM azaserine injected larval tissues viz, Mid gut (MG), Posterior silk gland (PSG), Fat body (FB) of *B. mori*. B) qRT-PCR analysis of the Cystine – glutamate transporter gene EAAT2 in control, 1 and 5 mM azaserine injected larval tissues. Data are shown as mean ± SEM (n = 6). †Significance was calculated by MANOVA and post-hoc test was done using SPSS11.5 and significance was considered at $P < 0.05$

Estimation of residual azaserine, cysteine and glutamate

Estimation of residual azaserine, cysteine and glutamate in larval tissues of *B. mori* were performed with a slight modification of earlier method of Vasilescu *et al*. (2013). Briefly, the tissue were extracted with methanol and were loaded on to the C-18 column (20 µL) with mobile phase that had two components A: phosphate buffer (0.01 M, pH = 3.60) and methanol in a ratio of 2:1 (v/v), while B: acetonitrile, in a final mixture of A:B = 75:25. Azaserine, cystine and glutamate were resolved on UV high pressure HPLC (Agilent Technologies, USA, Model 1120).

Glutamate-Cystine transporter gene expression

Glutamate-Cystine exchanger gene sequence was obtained through blast search of silk worm genome with data sourced from NCBI. Primers were designed and synthesized for Silkworm *tubulin* (Housekeeping gene) and *EAAT2* gene (Cystine-Glutamate Transporter) under study.

RNA Extraction and Real Time Quantitative Polymerase Chain Reaction (qRT-PCR) for EAAT2 *gene (Glutamate Transporter)*

Total RNA in the larval tissues viz mid gut, posterior silk gland and fat body were extracted using lysis buffer-β-ME mixture containing guanidine hydrochloride (Chromous RNA isolation kit). Samples were treated with RNase free DNase 1 to remove genomic DNA residual from RNA samples. 5 µg of total RNA was reverse-transcribed using 1 µg of oligo-dT primer, 2.0 µl of dNTP (2.5 mM), 10 U of ribonuclease inhibitor (RNasin) and 1.0 µl MMuLV RT (20 U/µl). The mixture was incubated at 42 °C for 1 h and the reaction was terminated by incubating the mixture at 92 °C for 2 min. The real-time PCR primers for target transcripts were designed using the complete cDNA sequences from NCBI (Table 1). A real-time run was taken on ABI Step-one Real Time PCR (Applied Biosystems, USA).

Briefly, real-time PCR reactions were performed with 50.0 µl reaction solution containing 2.0 µl first-strand cDNA, 2.0 µl of each forward and reverse primers, 25.0 µl of 2× SYBR green master mix and 20.0 µl of RNase-free H_2O_2. The thermal cycling conditions include 5 min at 94 °C, 5 s at 94 °C, 10 s at 55 °C, 10 s at 72 °C and 5 min at 72 °C for 40 cycles. The output from real time software was analyzed. The EAAT2 gene and tubulin transcript levels were estimated by using the formula $2^{\Delta\Delta Ct}$ where ΔCt represents the difference in Ct values between target gene and tubulin. RT-PCR data were normalized with tubulin mRNA levels and relative mRNA levels were expressed as $2^{\Delta\Delta Ct}$ values.

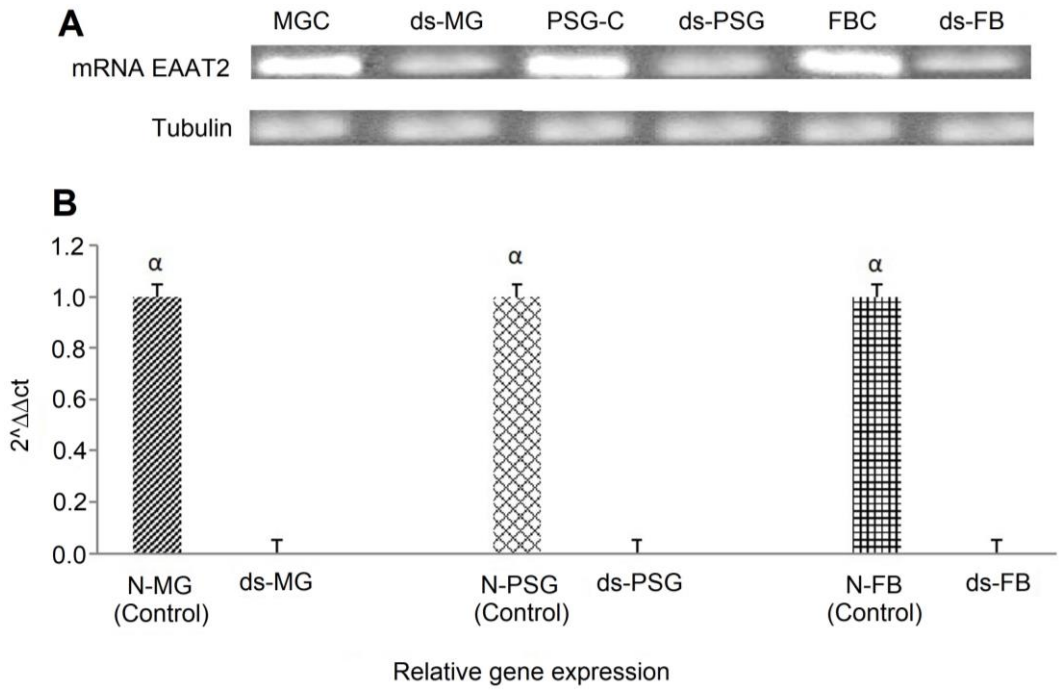

Fig 3 A) Representative agarose gel photograph from control and ds RNA injected larval tissues viz, Mid gut (MG), Posterior silk gland (PSG), Fat body (FB) of *Bombyx mori* . B) qRT-PCR analysis of the Cystine –glutamate transporter gene EAAT2 in control and ds RNA injected larval tissues Data are shown as mean ± SEM (n = 6). †Significance was calculated by MANOVA and post-hoc test was done using SPSS11.5 and significance was considered at $P < 0.05$

RNA interference studies on EAAT2 gene in B.mori

The dsRNA for Bm EAAT2 was synthesized using Chromous RNA kit. dsRNA of 40 µg was injected twice into each individual larvae with first injection on the 4 day of final instar and the second injection was at 2 h prior to the collection of tissues. Further the expression of the gene and role of RNAi was assessed by qRT-PCR.

Estimation of ATP

The tissue ATP was estimated with modification of earlier procedure by Korenberg, (1950). The principle involved for the above method is, ATP reacts with glucose in the presence of Mg^{2+} and hexokinase, forming glucose 6-phosphate which is further oxidized by glucose 6-phosphate dehydrogenase with simultaneous reduction of TPN. The rate of reduction of TPN is measured by observing the rate of change of optical density at 340 nm. In these coupled reactions, the final rate of reduction of TPN is equal to the rate of formation of ATP with or without creatine phosphate and expressed as µM of ATP formed/unit time.

Estimation of reduced glutathione

Glutathione was determined in larval tissues according to Boyne and Ellman (1972) based on 5-5'dithio (bis) nitrobenzoic acid (DTNB) reaction with compounds containing sulphahydryl groups. The final yellow colored reaction mixture was read at 412 nm and expressed as µM/mg tissue.

Determination of γ-glutmyl transpeptidase (GGT) activity

GGT activity was assayed according to Grisk *et al*. (1993), using γ- glutamyl-p-nitroanilide (GPNA) as donor substrate while glycyl-glycine as glutamate acceptor for the transpeptidation reaction. p-nitroaniline formed was read at 405 nm and expressed as mM substrate transformed/ml/min.

Statistics

Data are shown as the mean ± SD of six observations. Changes between groups were analysed by MANOVA and further tested by the bonferroni posthoc test, Kaplan-Meier test for survival analysis were assessed using statistical package for social sciences (SPSS) software (Huberty and Olejnik, 2006) and $p < 0.05$ was considered significant, statistically significant data are presented in the text.

Results

Survival of silk worm B. mori *injected with 1 and 5 mM azaserine*

V instar larvae were injected with 1 and 5 mM azaserine and the percentage of survival were noted after 24 h of injections. Fig. 1 shows significant increase in survival percentage in larvae injected with 5 mM in comparison to 1 mM.

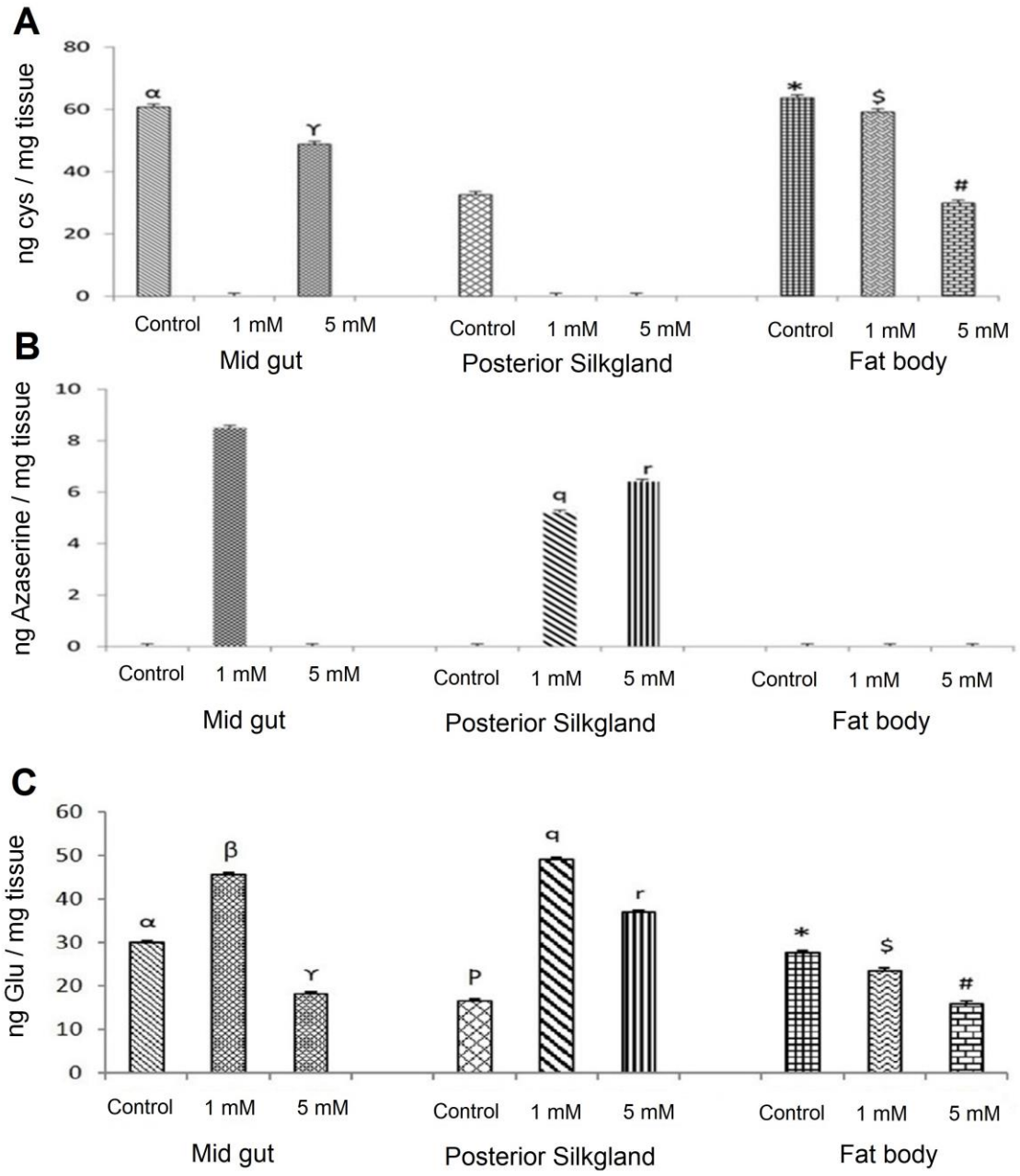

Fig. 4 Residual values (ng /mg tissue) of (A) cysteine (B) azaserine,(C) glutamate in control, 1 and 5 mM azaserine injected larval tissues viz, Mid gut, Posterior silk gland, Fat body from larvae of *B. mori* through HPLC. †Significance was considered at $P < 0.05$. Significance was represented as α, β and γ for mid gut, p ,q and r for posterior silk gland and *, $ and # for fat body

Tissue specific expression of Glutamate-Cystine transporter gene EAAT2 in B. mori

Larval tissues showed significant changes in expression of *EAAT2* gene, midgut tissue showed a significant decrease in expression of *EAAT2* with increase in concentration of azaserine (Fig. 2A and B) and in larval posterior silk gland there was a significant up regulation in 5 mM azaserine injected in contrast to 1 mM. Larval fat body showed an increase in expression of *EAAT2* with azaserine injections.

Silencing of Bm EAAT2 gene in B. mori *through RNAi studies*

Expression profiles of the gene EAAT2 in normal and dsBmEAAT2 RNA injected larvae in three tissues viz mid gut, posterior silk gland, fat bodies were compared. EAAT2 gene was found to be silenced in all the three tissues in dsBmEAAT2 RNA injected larvae (Fig 3A and B). The present results provided an evidence for the functionality of transporter gene EAAT2 in *B. mori*.

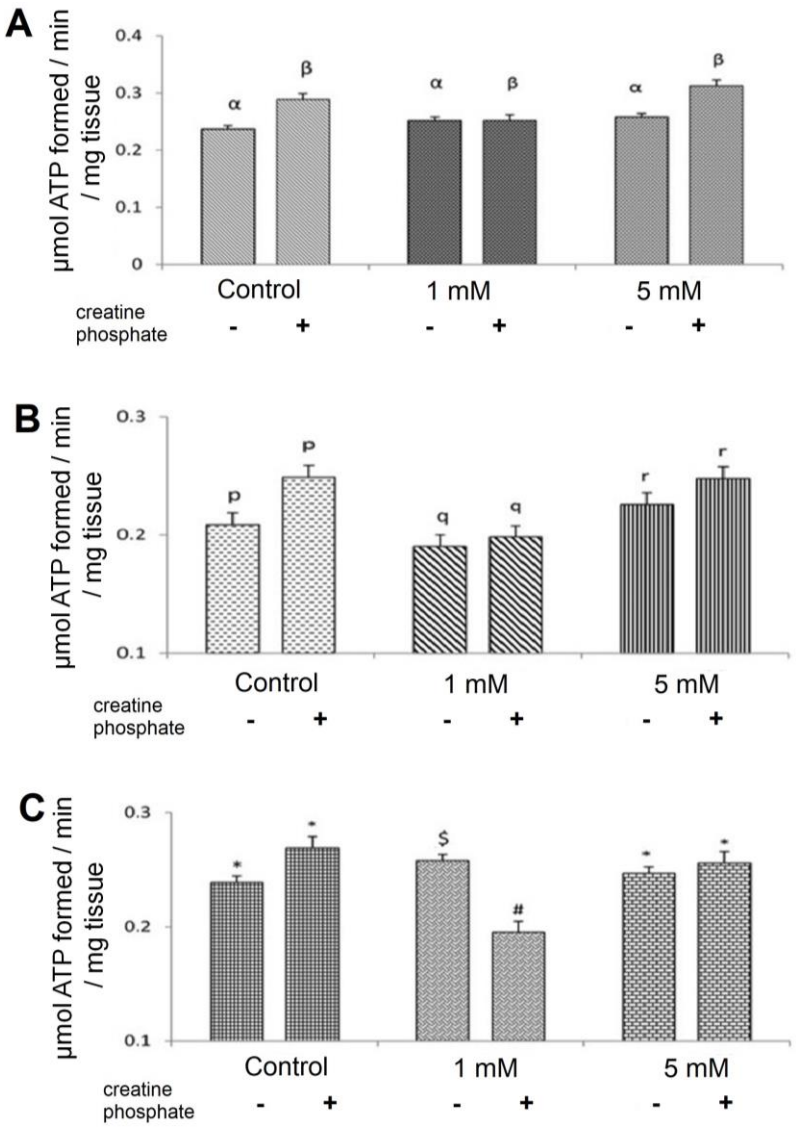

Fig. 5 Effect of Azaserine on ATP synthesis in (A) Mid gut (B) Posterior silk gland and (C) Fat body of larvae of *B, mori.* Values are expressed in terms of µM ATP formed/ mg protein/ min and represented as mean ± SEM (n = 6). † Significance was calculated by MANOVA and posthoc test was done using SPSS11.5 and significance was considered at $P < 0.05$. Significance was represented as α, β and γ for mid gut tissues, p, q and r for posterior silk gland tissue and *, $ and # for fat body tissues assayed in control, 1 and 5 mM azaserine injected larval groups + and - represent the ATP synthesized in presence and absence of creatine phosphate respectively

Residual concentration of Cystine, Azaserine and Glutamate in larval tissues of B. mori

Residual concentration of amino acid Cystine in larval tissues injected with 1 and 5 mM azaserine were assessed using HPLC agilent zorbax column (C-18). Significant decrease in cystine was observed in mid gut of larvae injected with 5 mM azaserine whereas traces of cystine were observed at 1 mM azaserine injected. Cystine was undetectable in posterior silk gland of treated larvae. Larval fat body showed significantly decreased concentrations of cystine with increased concentrations of azaserine (Fig. 4A). The residual concentrations of azaserine were significantly evident only in mid gut of larvae injected with 1 mM azaserine whereas undetectable in mid gut of larvae injected with higher dosage. Residual concentration of azaserine in posterior silk gland increased with azaserine dosages (Fig. 4B). However fat body tissues were found to have no azaserine irrespective of concentrations. In the larvae injected with 1 mM azaserine showed a significant increase in the values of glutamate in mid gut and posterior silk gland, however fat body showed an insignificant change in glutamate levels. In the 5 mM azaserine injected larval tissues, mid gut and fat bodies showed a decrease in glutamate levels while elevated levels were observed in posterior silk gland (Fig. 4C). Interestingly ds RNA injected larvae showed insignificant values of L-glutamate.

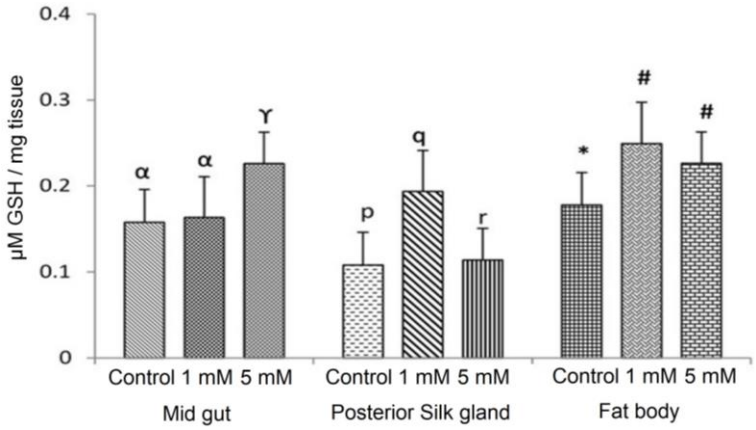

Fig. 6 Effect of Azaserine on Glutathione (GSH) in Mid gut, Posterior silk gland and Fatbody of larvae of *B. mori*. Values are expressed in terms of µM GSH/mg tissue and represented as mean ± SEM (n = 6). † Significance was calculated by MANOVA and posthoc test was done using SPSS11.5 and significance was considered at $P < 0.05$. Significance was represented as α, and γ for midgut tissues p, q and r for posterior silk gland tissue and * and # for fat body tissues assayed in control, 1 and 5 mM azaserine injected larval groups

Effect of azaserine on ATP formation in B. mori

Tissues were assayed for ATP formation in the presence (+) and absence (-) of creatine phosphate. The larval mid gut and posterior silk gland showed a significant increase in ATP formed in 5 mM azaserine injected while 1 mM azaserine injected larval tissues did not show ATP formation in the presence of creatine phosphate. In the larval fat body tissue ATP formation showed a significant decrease in 1 mM injected and no significant changes in higher dosage (Fig. 5).

Effect of azaserine on Glutathione (GSH) levels in tissues of B. mori

Glutathione is an antioxidant molecule. The larval mid gut tissue reveals significant increase in GSH activity at higher dosage while the posterior silk gland showed a significant increase in GSH activity in lower dosage. A significant increase in GSH activity was observed in fat body at both the dosages of azaserine injected (Fig. 6).

Effect of azaserine on Gamma Glutamyl transpeptidase (GGT) levels in tissues of B. mori

GGT being membrane bound and externalizes GSH, significant inhibition of GGT activity by 7 folds was noted in mid gut tissues at both the dosages. The larval posterior silk gland showed inhibition only in 1 mM injected while the 5 mM injected showed no change, contrastingly the fat body showed significant elevation in GGT activity at both the dosages of azaserine injected (Fig. 7).

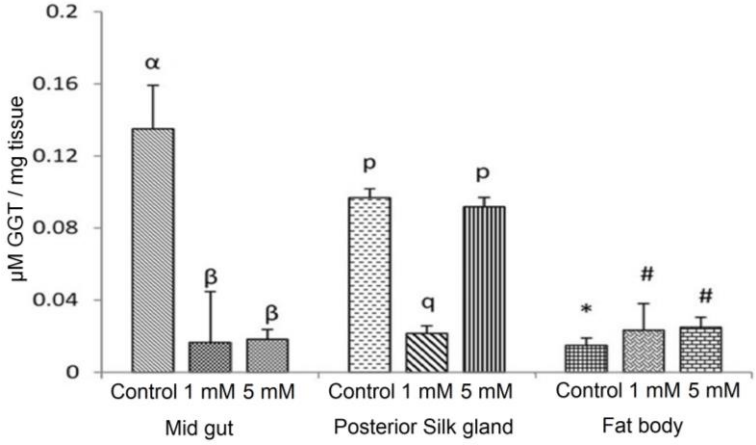

Fig. 7 Effect of Azaserine on Gamma glutamyl transpeptidase (GGT) in (A) Mid gut (B) Posterior silk gland and (C) Fatbody of larvae of *B. mori*. Values are expressed in terms of µM GGT/mg tissue and represented as mean ± SEM (n = 6). †Significance was calculated by MANOVA and posthoc test was done using SPSS11.5 and significance was considered at $P < 0.05$. Significance was represented as α and β for mid gut tissues, p and q for posterior silk gland tissue and * and # for fat body tissues assayed in control, 1 and 5 mM azaserine injected larval groups. † (significance between control and among dosages is considered and not between types of tissues for all the assays)

Survival Analysis

Survival analysis among the test groups (Dosages) were assessed using Kaplan-Meier test. The outcomes of the analyses show significant difference in survival of larvae injected with 1 mM and 5 mM dosages of azaserine. The results show better survival at higher dosages of injections than lower dosages. The average survival post 24 h was about 17% with 1 mM injections and 30% with 5 mM injections (Fig. 8).

Discussion

Azaserine is a known analogue of glutamine and often used as a drug. As the drug dosages become important in different stages of treatment, it is imperative that higher dosages have severe impact compared to lower dosages and the LD_{50} is a norm in deciding admissible drug dosages, however the present study has the contrasting result of lesser survival of larvae at lower dosages and greater survival at higher dosages, as such the concept of LD_{50} is not relevant in the present context. Azaserine inhibits GGT irreversibly (Hsu, 1980), our results have shown that GGT is inhibited by azaserine at both dosages in mid gut. In the posterior silk gland inhibition was evident at lower dosage and higher dosage has no effect on GGT activity contrary to these the fat body had significant elevation in GGT activity at both dosages. These observed changes could be due to induced changes in redox status (Garama *et al.,* 2015) and the GSH catabolism along with intracellular and extracellular cystine pool as reported (Paolicchi, 2002). In *Sporodoptera littoralis*, a lepidopteran, the increased GGT activity is due to the OS induced by the spores of *Baveria bassiana* (Mirhaghparast *et al.,* 2013). In our studies azaserine has impacted GGT differently with different dosages in the tissues studied. However the mechanistic of activation or inhibition of GGT was not attempted in the present study.

As GSH is a non-enzymatic antioxidant, it becomes imperative in understanding GSH metabolism in stressed state. Our results showed an increase in GSH activity in mid gut at higher dosage, at lower dosage in posterior silk gland, both dosages in fat body and these changes are tissue specific. These outcomes could be due to azaserine induced redox changes. In phytophageous insect such as *Lymantria dispar* have higher GSH activity in the mid gut in order to defend against pro oxidant effect of plant allelochemicals and xenobiotics (Maturga *et al*., 1997). Our results with mid gut are concurrent with the above as GSH changes are imminent for survival of insect species (Buyukguzel

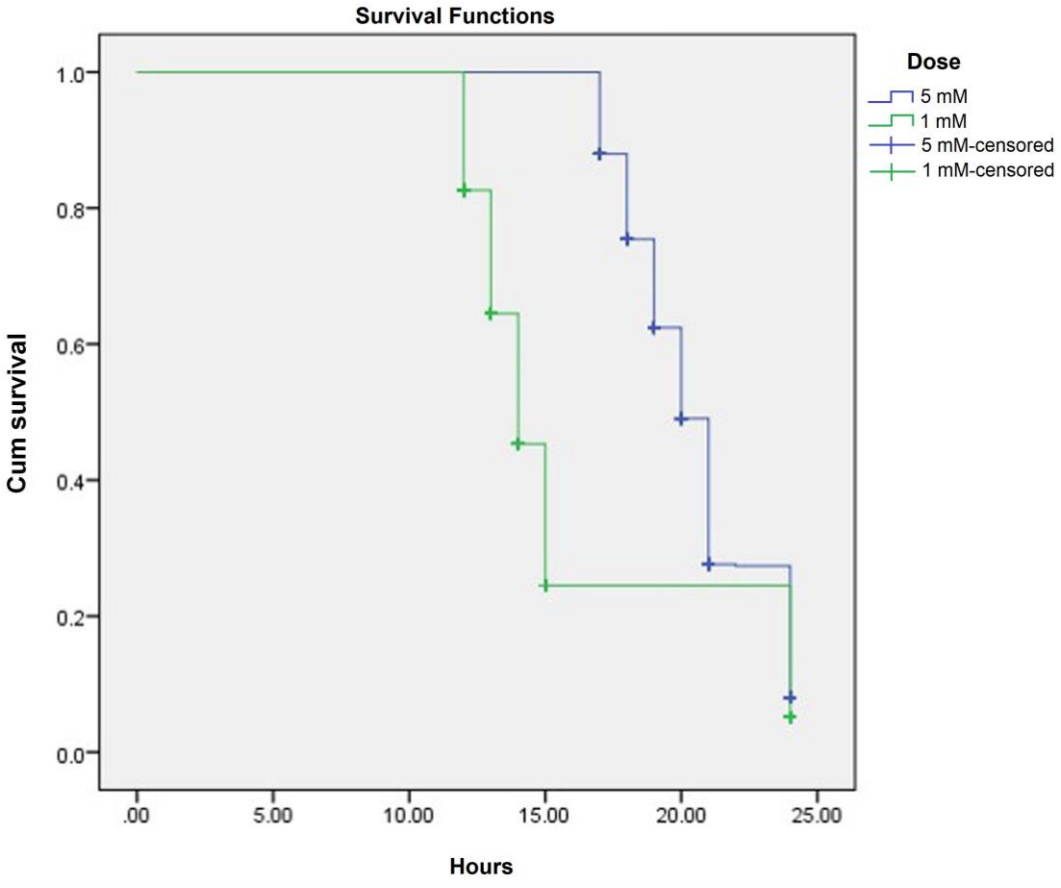

Fig. 8 Kaplan-Meier statistical analyses depicting significant differences in survival between dosages of azaserine in *B. mori*

and Kelender, 2007; Poupardina *et al*., 2008; Vijayvel and Balasubramanian, 2009; Ahemad, 2011; Fahmy, 2012) and also GSH can non-enzymatically react with different ROS and can be a free radical scavenger (Winterbourn, 1994).

Cystine ($Cyss^-$) is an oxidized, whereas cysteine (2xcys) is reduced form. In GSH homeostasis, intracellular cysteine is a part of tripeptide GSH and externalized by GGT. During the process cysteine is converted to cystine and can make an entry either in acidic or alkaline forms, whereas cystine in neutral form could arise due to extracellular protons, thus makes cystine impermeable (Bannai and Kitamura, 1981). Interestingly our results for the first time show no traces of cystine in the tissues that showed residual azaserine, clearly emphasizing azaserine cysteine transport in silk worm *B. mori* and further emphasized by expression of EAAT2.

Excitatory amino acid transporters co transports glutamine with cysteine or aspartate (Shankar *et al*., 2001) and uptake of cysteine and glutamate are mutually competitive, EAAT2 inhibitors also inhibit cysteine uptake (Chen and Swanson, 2003), however the increased residual glutamate concentration in certain tissues can be attributed due to co transport mechanism involving Na^{2+} even though EAAT2 was inhibited. Our results of EAAT2 inhibition by azaserine are consistent with the above particularly in mid gut and posterior silk gland. Interestingly the cysteine uptake has been totally inhibited in tissues showing residual azaserine however EAAT2 expressions was up regulated in fat body. Insect EAAT's have been cloned and compared to mammalian EAAT's and they share high degree of amino acid identity, comparable kinetics and pharmacological properties (Donly and Caveney, 2002). Silk worm *B. mori* EAAT2 amino acid sequence shares 83% homology with lepidopteran insect *Chilo suppressalis* especially in fat body and mid gut (Xu *et al*., 2015). Larval neutral amino acid transporters show different dependency on pH and an increased uptake was seen at alkaline pH (Hannigan, 1993; Sacchi *et al*., 1994). The injection of azaserine can induce changes in the pH in insect system as the drug is itself acidic in nature and may impair glutamate transport as indicated by down regulation of EAAT2 (Castanga, 1997). In the present study down regulation of EAAT2 transporter in posterior silk glands injected with both dosages can be attributed to the presence of residual azaserine in glandular epithelium.

The expression of EAAT2 gene was silenced using a double stranded RNA (dsRNA) that degrades the target mRNA (Fire *et al*., 1998). Our studies confirm the presence of EAAT2 gene in *B. mori* and further established with silencing of the same gene by introducing ds RNA. Several RNAi studies have been performed in *Bombyx mori* embryos and larvae to understand the role of various genes in developmental process and immunity (Quan *et al.,* 2002; Rajagopal *et al*., 2002). The mode of dsRNA introduction, the quantity or dosages of dsRNA have shown variations with different tissues of *Bombyx mori* (Terenius *et al*., 2011). In the present study 80 µg of dsRNA was injected to bring about effective silencing of the gene.

Biological form of energy is the ATP molecules that define the status of cells ability to survive or to succumb to the critical conditions of any form of stress or disease. Our results show an increased ATP levels in tissues injected with higher dosage of the drug indicating the rejuvenation of cellular energy synthetic machinery but not in lower dosage. The survival of the system is thus energy dependent.

Conclusion

The survival may be a cumulative outcome of all above factors driven by the ATP availability. Our results indicate a better chance for survival in larvae injected with higher dosages may be due to rejuvenation of ATP formation that was not observed in lower dosages. We presume that a higher dosage of azaserine facilitate high turnover of cysteine enabling better survival. The present work emphasizes the role of EAAT2 in externalization of azaserine that aids better survival of larvae that received higher dosage of the drug.

Compliance with Ethical Standards

N/A

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgement

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