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Assessment of electron beam irradiation induced proteomic changes and its effect on the development of silkworm, *Bombyx mori* (Bombycidae: Lepidoptera)

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Abstract The present study has been designed to determine the hemolymph protein changes induced by EBI using Microtron (ranges from 20 to 100 Gy) on the 5th instar larvae of silkworm, *Bombyx mori* using SDS–PAGE. The EBI did not caused any impact on 5th instar larval hemolymph proteins, however a significant reduction of pupal hemolymph proteins such as lipophorin (250 kDa), vitellogenin (180 kDa), storage protein (76–80 kDa) and a 30 kDa protein was observed through SDS–PAGE and densitometry analysis. These proteins are known to play a crucial role in various developmental processes including transport of lipids, as amino acid reservoir for providing precursors for egg and cuticle formation and immunity of *B. mori*. Further, a decrease of antioxidant enzymes such as Superoxide Dismutase (SOD) and Catalase (CAT) in the EBI treated larval and pupal hemolymph was observed. In addition, a negative influence on growth characteristics and appearance of pupal deformity was noted. This may be due to damage in hemolymph proteins, when the larvae were exposed to EBI at >80 Gy.

Introduction

High energy electrons are used for various research and development (R&D) applications such as polymer processing, surface curing, sterilization of medical products and food irradiation in an eco-friendly manner (Siddappa et al., 1998; Machi, 2008; Kim et al., 2010; Sanjeev, 2012). Recently EBI

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is demonstrated by many researchers for managing insect pests, as an alternative approach to insecticide and gamma radiation (Moon et al., 2010; Koo et al., 2011, 2012; Wang et al., 2012; Hallman, 2013; Yun et al., 2014). Further they reported the occurrence of DNA damage and defect in the growth parameters of various insect pests such as *Spodoptera litura*, *Plutella xylostella*, *Myzus persicae* and *Liposcelis paeta* by the effect of EBI treatment. Recently, Wasielewski et al. (2014) stated that enhanced UV-B radiation can reduce body mass and fat body content, cause deformities and increase mortality in female adults of the solitary bee *Osmia bicornis*. However upon radiation exposure the role of hemolymph proteins with respect to the developmental process is unclear, therefore a detailed study is needed to analyze the effect of EBI on hemolymph proteins and its detrimental impact on the metamorphosis of insects.

Studying the hemolymph protein profile of the insect is important because it plays a pivotal role in different physiological and developmental processes (Krishnan and König, 2011). Hemolymph proteins such as lipophorin, Vitellogenin, Storage protein and 30 kDa protein are synthesized from larval peripheral fat body tissue, released into hemolymph for the transport and stored in the pervisceral fat body tissue. Then the stored proteins are utilized throughout pupal to adult transition (Vanishree et al., 2005). Among the hemolymph proteins, lipophorin acts as a carrier of lipid, nutrients and pigments for developing embryo and silk gland respectively (Ziegler and Van Antwerpen, 2006). Vitellogenin is a female specific protein, utilized for egg production (Singh et al., 2013). Storage protein (Sp) is a multifunctional protein involved in cuticle development, anti-apoptosis, egg development and supply nutrient for pupal to adult transition (Tojo et al., 1980; Vanishree et al., 2005; Krishnan and König, 2011). The 30 kDa protein belongs to the lipoprotein family and acts as an anti-apoptotic agent (Kim et al., 2003; Pakkianathan et al., 2014). So, these hemolymph proteins have a significant role on growth and reproduction of insects.

Hence, the proteomics study is an ideal and a better technique to understand the physiology and adaptation of insects than genomics study. In this context, our study aims to apprise the EBI induced proteomic changes and its effect on the metamorphosis of silkworm, *B. mori*. In this study, we tried to shed some light on the possible role of hemolymph proteins on the growth of silkworm and applicable doses of EBI in future applications.

Materials and methods

Insect rearing

Disease free eggs of domesticated silkworm, *B. mori* (the cross-breed race, Tamil Nadu White X NB4D2) were obtained from the Government Grainage Centre, Tiruchirapalli, India. The eggs were incubated for a successful hatch at 27 ± 2 °C and 75 ± 5% relative humidity. Newly hatched larvae were fed with chopped tender leaves of the mulberry variety (MR2) until 3rd instar and with coarse leaves upto the last instar (Nirmala et al., 1999). The 5th instar larvae were transported to Microtron Centre, Mangalore University, Mangalore, for irradiating the larvae with electron beam using Microtron (Designed by Raja Ramanna Centre for Advanced Technology, Indore).

Electron beam irradiation of B. mori larvae using Microtron

Two day old 5th Instar larvae (10 no's/sachet) of the silkworm were kept inside the polypropylene bag (BOPP, 25 μ, 6 × 6 cm) ahead of the beam exit point at a distance of 30 cm and exposed to whole body irradiation at 20, 40, 60, 80 and 100 Gray (Gy) using a Microtron (8 MeV pulsed electron beam accelerator) at room temperature (25 ± 2 °C). For each dose, the treatment was performed in triplicate. The absorbed doses were determined using Chemical Fricke dosimetry as mentioned in Acharya et al. (2009). The pulsed beam electron acceleration from the Microtron was observed with a maximum pulse current of 50 mA and a pulse duration of 2.5 μs as described by Siddappa et al. (1998). Two day old 5th Instar larva of the silkworm without irradiation was kept as a control group.

Collection of hemolymph

Hemolymph was collected after two hours from EBI treated and control groups of silkworm by making a slit in a proleg as described by Pakkianathan et al. (2012). In the case of pupa, the hemolymph was collected by making a prick on the abdominal cuticle using a sharp pin. Hemolymph that dripped from the wound without external pressure was collected in an Eppendorf tube which was pre-rinsed with phenylthiourea solution (1 mg/ml) to prevent tyrosinase activity. The collected samples were centrifuged at 10,000 rpm in 4 °C for 10 min (Table top centrifuge, Sigma, Germany) to remove hemocytes and cell debris. The hemolymph sample was stored at −80 °C until further use (SANYO deep freeze, Japan).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Total hemolymph proteins from both EBI treated and control larva and its pupa were quantified (Bradford, 1976) and subjected to SDS–PAGE consisting of 12% separating and 4% stacking gels (Laemmli, 1970). Approximately 30 μg of protein was taken from each sample and mixed with an equal volume of 1× sample loading buffer (0.125 M Tris–HCl pH-6.8, 4% SDS, 10% β-mercaptoethanol, 20% Glycerol and 0.02% bromophenol blue). The mixture was boiled at 95 °C for 5 min and loaded in respective lanes. SDS–PAGE run was performed until bromophenol blue front reached the end of the gel (50 V and 70 V were used to run stacking and separating gels respectively). Later, gels were stained with Coomassie brilliant blue R-250 staining solution (0.1% Coomassie brilliant blue R-250 in 40% methanol and 10% glacial acetic acid) for 6 h. The stained gels were rinsed with double distilled water (DDH_2O) and placed in a destaining solution (40% ethanol: 10% glacial acetic acid) until the appearance of clear bands on the gel. Protein size was estimated according to the standard protein molecular weight marker (Ruler plus unstained Protein Ladder) procured from Thermo scientific, USA. The intensity of CBB stained protein bands was analyzed quantitatively for larval and pupal hemolymph protein between EBI treatment and control using densitometry ImageJ software (Rasband, 1997).

Estimation of antioxidants

Aliquots of 100 µl hemolymph samples from both two day old 5th instar EBI treated and control larval and pupal groups were processed for the measurement of two major antioxidant enzymes (SOD and CAT). SOD was assayed according to the method of Marklund and Marklund (1974) with slight modifications. A fraction of hemolymph sample (0.1 ml) was mixed with 0.25 ml of absolute ethanol, 0.15 ml of chloroform and 1 ml of DDH_2O. The mixture was centrifuged at 2500 rpm for 15 min and the supernatant was mixed with 2 ml of 0.1 M Tris EDTA buffer (pH 8.2). At the time of spectrophotometer analysis, pyrogallol (0.5 ml) (12.6 mg in 50 ml of 50 mM Tris HCl Buffer (pH 7.4)) was added to the mixture. The rate of autoxidation of pyrogallol was noted at an interval of 1 to 3 min at 420 nm.

CAT was assayed by the method of Sinha (1972) with minor modifications. A fraction of hemolymph (0.1 ml) was mixed with 0.5 ml of hydrogen peroxide (60 mM H_2O_2), 1.0 ml of phosphate buffer (0.01 M, pH 7.0) and 0.4 ml of DDH_2O. To the mixture, 2.0 ml of dichromate/acetic acid reagent was added after 15, 30 and 60 s of incubation time at room temperature. To the control tube, the hemolymph was added after the addition of acid reagent. The tubes were then heated for 10 min in a boiling water bath until color development and it was read at 570 nm. The activity of catalase was arrived at from the amount of hydrogen peroxide consumed and was expressed as µmoles of hydrogen peroxide consumed/mg protein/min.

The effect of EBI on growth characteristics of B. mori larvae

EBI treated larval growth characteristics were investigated upto adult stage. Growth characteristics such as larval period, survivability, spinning (cocoon formation), pupal period, pupal mortality and adult emergence were observed throughout the life cycle of *B. mori* in each dose to access the impact of EBI. Also egg formation in the ovaries of pupa developed from the irradiated larva was observed and counted under a light microscope (Hund Wetzlar, Germany).

Statistical analysis

All values were expressed as mean ± SD and statistically analyzed by a one-way analysis of variance (ANOVA) and significance was analyzed among control and EBI treatment with a Tukey's pairwise comparisons using PAST version 3.05 (Hammer et al., 2001). Differences were considered significant if $P < 0.05$.

Results

Electrophoresis investigation of EBI treated larval and its pupal hemolymph proteins

In the present study, the total hemolymph protein (~30 µg) from both EBI treated and control samples of larva and its pupa were analyzed by 12% SDS–PAGE. The SDS–PAGE analysis for larval hemolymph proteins showed around 7 to 9 predominant coomassie blue (CBB) stained protein bands at different molecular weights, ranging from 15 to 250 kDa in both control and treated groups. However, no significant difference between control and treated larval groups was noted in the major protein bands designated as lipophorin, storage protein and 30 kDa proteins (Fig 1). An elevation of protein peptides at the 40 kDa region was noticed in the irradiated larval hemolymph (arrow indicates the presence of a peptide in Fig 1) when compared with the control. Densitometry analysis revealed that the 40 kDa band was significantly increased in all doses of EBI when compared with the control but it was less significant among the treatments (Table 1).

Surprisingly, the SDS–PAGE analysis for pupal (six day old pupa) hemolymph proteins showed a reduction in their CBB staining intensity of vital protein bands resolved at 250 (lipophorin), 180 (vitellogenin), 74–80 (storage protein; SP1,2 and 3) and 30 kDa (anti-apoptotic protein) when compared with the control (Fig 2). The reduction in the CBB staining intensity of pupal hemolymph proteins was quantitatively validated by densitometry analysis (Table 1). The control pupa (lane PC in Fig 2) clearly showed the presence of major hemolymph proteins in significant quantity. These proteins were previously isolated, purified and characterized by our laboratory co-workers using specific antibody and mass spectrometry analysis (Vanishree et al., 2005; Pakkianathan et al., 2012, 2014; Singh et al., 2013; Pratheep, 2013).

Antioxidant enzyme level in EBI treated larval hemolymph of B. mori

Antioxidants are an important agent in all cellular organisms because they inhibit the oxidation of other molecules and prevent free radicals. The major anti-oxidants to quench the free radicals are SOD and CAT (Halliwell et al., 2000). In the present study, SOD and CAT activities were significantly ($P < 0.05$) reduced in *B. mori* larval hemolymph following exposure to EBI at higher doses (80 and 100 Gy) and less significant in other doses in comparison with the control (Fig. 3 (A) and (B) as revealed by Tukey's pairwise comparisons

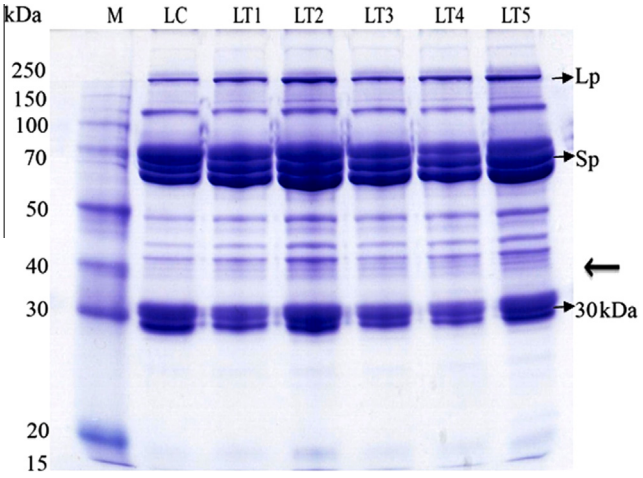

Figure 1 SDS–PAGE pattern of larval hemolymph proteins (two days old 5th instar larvae) of control and EBI treated silkworm, *B. mori* (LC-larvae control; LT1-20 Gy; LT2-40 Gy; LT3-60 Gy; LT4-80 Gy; LT5-100 Gy. Lane M stands for Protein ladder purchased from Thermo scientific, USA).

Table 1 Quantitative analysis of hemolymph protein bands from larva and pupae of Silkworm, *Bombyx mori* using densitometry.

Stage of hemolymph sample	Name of the protein band	Doses of EBI in Gray (Gy)						One way ANOVA analysis	
		Control	20	40	60	80	100	*F*	*P*
Relative density of larval hemolymph protein†	40 kDa	1.0 ± 0.0a	2.33 ± 0.08a	5.46 ± 0.41a	2.38 ± 0.16a	2.17 ± 0.13a	2.35 ± 0.13a	1.175	NS*
Relative density of Pupal hemolymph protein†	Lipophorin (Lp)	1.0 ± 0.0a	1.02 ± 0.00a	0.76 ± 0.00b	0.66 ± 0.01c	0.61 ± 0.02c	0.35 ± 0.00d	937.7	<0.05
	Vitellogenin (Vg)	1.0 ± 0.0a	0.90 ± 0.00b	0.44 ± 0.01c	0.47 ± 0.02c	0.26 ± 0.02d	0.20 ± 0.03d	591.3	<0.05
	Storage protein (Sp)	1.0 ± 0.0a	0.77 ± 0.00b	0.58 ± 0.00c	0.40 ± 0.00d	0.41 ± 0.00d	0.43 ± 0.01d	4699	<0.05
	30 kDa protein (30 kDa)	1.0 ± 0.0a	0.90 ± 0.05a	0.49 ± 0.02b	0.52 ± 0.02b	0.48 ± 0.02b	0.59 ± 0.05b	74.74	<0.05

Means followed by same letters in rows are not significantly different at $P = 0.05$ using Tukey's pairwise comparisons. Degree of freedom for every variable is 17. The P – value indicates the significance at <0.05 using One way ANOVA analysis. NS-indicates non-significant. NS* indicates the unequal variances show the significance at <0.05 using Welch F test.
† All data are means (±SD).

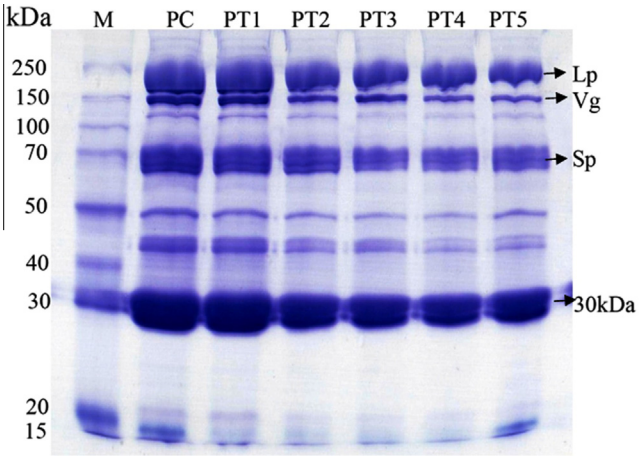

Figure 2 SDS–PAGE pattern of pupal hemolymph protein (Six days old pupa developed from control and EBI treated larvae) of silkworm, *B. mori* (PC-pupae control; PT1-20 Gy; PT2-40 Gy; PT3-60 Gy; PT4-80 Gy; PT5-100 Gy. Lane M stands for Protein ladder purchased from Thermo scientific, USA).

and a one way ANOVA analysis. Similarly a reduction of SOD and CAT levels was significantly ($P < 0.05$) noted in the pupal hemolymph of *B. mori* (Fig. 4(A) and (B). This result suggests that EBI stress has the potential to alter the antioxidant enzyme level in the larval hemolymph of *B. mori*. Moreover, the mRNA transcript of SOD and CAT enzymes needs to be studied for better understanding of oxidative stress produced by EBI.

Effect of EBI on the growth characteristics of B. mori

In the present study, the effect of EBI on the larvae and its development upto adult emergence has been investigated. A one-way ANOVA with Tukey's pairwise comparison analysis clearly revealed that the EBI doses are not significantly affecting the larval period (in days), spinning, and the pupal period (in days) (Table 2). However, larval survivability was significantly affected by EBI ($F = 17.4$, $P < 0.05$) than the control. The pupa developed from the irradiated larvae showed significantly higher mortalities than control at all doses ($F = 159.1$, $P < 0.05$).

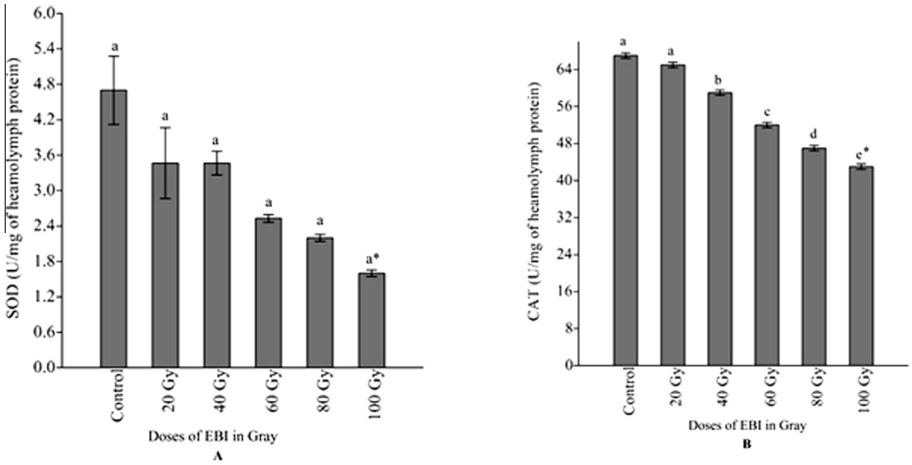

Figure 3 (A) Estimation of Superoxide Dismutase (SOD) in the hemolymph proteins from EBI treated two days old 5th instar larvae of silkworm, *B. mori*. (B) Catalase (CAT) level in the hemolymph proteins from EBI treated two days old 5th instar larvae of silkworm, *B. mori*. The same superscript over error bars means values are not significantly different at $P > 0.05$. *100 Gy of EBI showed more significant in the reduction of enzymes level when compared with control.

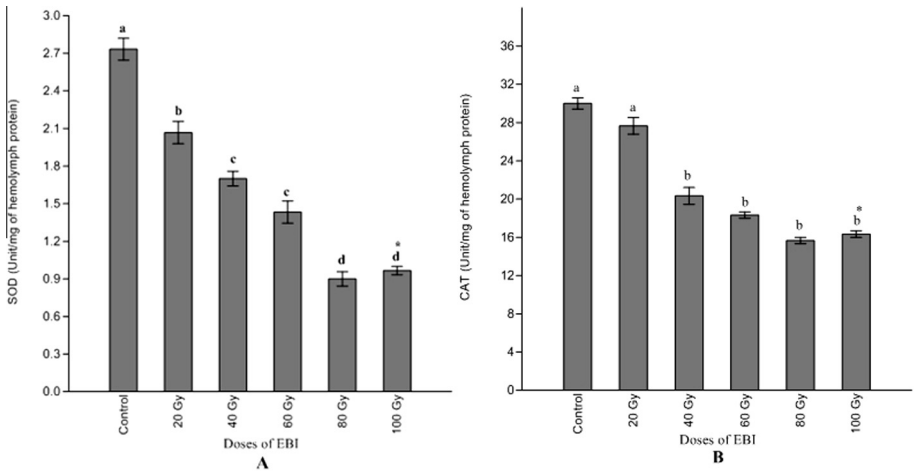

Figure 4 (A) Estimation of Superoxide Dismutase (SOD) in the 6 days old pupal hemolymph proteins of silkworm, *B. mori*. (B) Catalase (CAT) level in the 6 days old pupal hemolymph proteins of silkworm, *B. mori*. The same superscript over error bars means values are not significantly different at $P > 0.05$. *100 Gy of EBI showed more significant in the reduction of enzymes level when compared with control.

Table 2 Effect of EBI on growth characteristics of silkworm, *B. mori*.

Growth characteristics (mean ± standard deviation (SD)[†]	EBI (doses in gray)							
	Control	20	40	60	80	100	One Way ANOVA	
No. of individuals in triplicate	30	30	30	30	30	30	*F*	*P*
Larval longevity in days	7.00 ± 0.00a				6.60 ± 0.57a	6.00 ± 1.00a	0.5	NS
Larval survivability	100.00 ± 0.00a				97.30 ± 0.57b	96.00 ± 1.00b	17.4	< 0.05
Spinning	100.00 ± 0.00a	90.00 ± 5.0a	90.00 ± 5.0a	88.00 ± 6.08a	87.66 ± 5.50a	86.66 ± 5.50a	2.757	NS
Pupal longevity in days	8.00 ± 0.00a			7.60 ± 0.57a		6.60 ± 0.57a	1.5	NS
Pupal mortality	0.00 ± 0.00a	45.00 ± 5.00b	51.00 ± 5.29b	69.00 ± 6.55c	73.66 ± 1.52 c	87.00 ± 2.64d	159.1	< 0.05
No. of eggs/pupa	293.66 ± 34.93a	175.00 ± 5.00b	85.33 ± 9.50 c	29.33 ± 5.13 d	14.33 ± 3.05 d	7.66 ± 0.57e	1470	< 0.05
Adult emergence	95.70 ± 1.00a	36.33 ± 1.52b	23.33 ± 2.88 c	12.66 ± 0.57 d	9.30 ± 1.52e	0.0 ± 0.00e	172.2	< 0.05

Means followed by same letters in rows are not significantly different at $P > 0.05$ using Tukey's pairwise comparisons. Degree of freedom for every variable is 17. The *P* – value indicates the significance at < 0.05 using One way ANOVA; NS-indicates non-significant.

[†] All data are means (±SD).

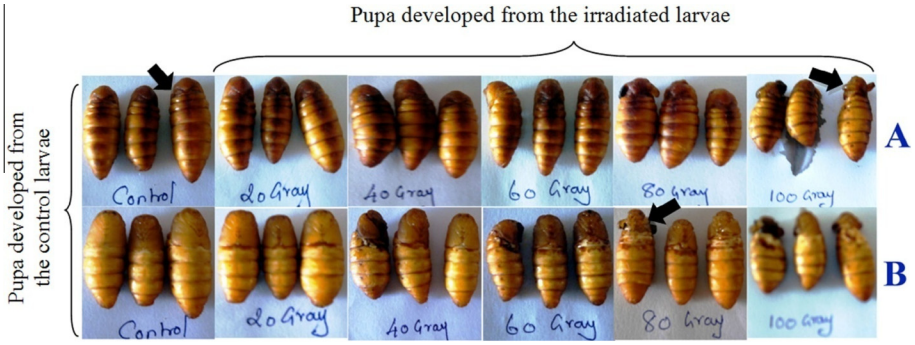

Figure 5 Developmental abnormalities in the six days old pupa emerged from EBI treated two days old 5th instar larvae of silkworm, *B. mori* ((A) Dorsal and (B) Abdominal view).

In addition to the pupal mortality, as confirmation the presence of pupal deformity (six day old pupa developed from EBI treated larvae) was also noted at 80 and 100 Gy of EBI (Fig. 5). Occurrence of deformity in the head region of the pupa was indicated by an arrow on the right side in both dorsal (A) and ventral (B) views. On the other hand, absence of deformity in the pupa developed from control larvae is marked by an arrow on the left side (Fig. 4). Moreover, a significant reduction in the egg formation per pupal ovarioles ($F = 1470$, $P < 0.05$) and adult emergence ($F = 172.2$,

$P < 0.05$) was increased while increasing the doses of EBI. No adult emerged from EBI treated larvae at 100 Gy. Therefore, our experiment confirms that the dose of 100 Gy has a lethal effect on the larvae of *B. mori*. Further, we propose that <40 Gy of EBI is a safe dosage for future silkworm research.

Discussion

Proteomics is emerging as a systems-level identification tool for analyzing cellular functions and their regulatory mechanisms better than genomics and transcriptomics (Mallick and Kuster, 2010). Several studies reported that any kind of stimulus/radiation stress on the insect immediately reflects on the hemolymph protein expression (Lin et al., 2001; Lopez-Martinez et al., 2008; Meng et al., 2009, 2010; Hou et al., 2010). Similarly, the hemolymph protein expression at 40 kDa was observed in the EBI treated larvae. The pupal hemolymph proteins (six day old pupa developed EBI treated 5^{th} instar larvae) including lipophorin, vitellogenin, storage protein and 30 kDa proteins were significantly reduced with respect to radiation doses.

Anti-oxidants play a major role in the feeding larva than in pupal stage as reported by Krishnan and Kodrik (2006), because antioxidant enzymes are up-regulated to protect the body against ROS generated by the ingested pro-oxidant allelochemicals. This may be a reason for the higher levels of SOD and CAT in the control larva than control pupa of *B. mori*. Further, we have observed that the gradual decrease of SOD and CAT in the treated larval and pupal groups (20–100 Gy) than the control. Similarly, Azzam et al. (2012) reported that the protective mechanism of antioxidants may not be sufficient to cope with stress produced by ionizing radiation. The present study indicates the harmful effects of EBI doses (>80 Gy) on anti-oxidant enzymes in the treated larval and its pupal hemolymph leads to lengthy oxidation of hemolymph proteins. We speculate that oxidative stress is a causative agent of the decline of vital protein concentrations in the pupal heamolymph of *B. mori*.

While coming to growth characteristics, EBI did not cause immediate mortality in the larvae in support of previous reports (Koo et al., 2011, 2012; Yun et al., 2014). However, the severe effect of EBI on larval survivability, pupation, egg formation in the ovarioles and adult emergence of *B. mori* has been observed. Similarly, several scientists reported a defect in adult emergence from the EBI treated pupa of others insects such as *P. xylostella*, *M. persicae* and *L. paeta* at 200 Gy (Moon et al., 2010; Koo et al., 2011; Wang et al., 2012). Recently, Yun et al. (2014) reported no adult emergence from the irradiated *Spodoptera litura* larvae at 150 Gy. Similar to the above report, we have also observed inhibition of adult emergence from EBI treated larvae at 100 Gy. Recently, Hiyama et al. (2012) reported that Fukushima dai-ichi nuclear power plant accident has caused developmental abnormalities in the insect, *Zizeeria maha*. Similarly in our study, the pupa developed from the irradiated larvae showed a notable deformity in the head region when the larvae were exposed >80 Gy. From this study, we have observed an increase in mortality and morphological deformities, especially when EBI doses exceed above >80 and 100 Gy. However, no evidence presents that the effect EBI on larval hemolymph protein and the hemolymph protein impact on growth characteristics of *B. mori*. Schloter (1985) stated that the decrease of hemolymph protein including storage protein might inhibit the metamorphosis of the insect, *Epilachna varivestis* after injection of azadirachtin. Silencing of apolipophorin-III gene expression caused an abnormal adult morphological phenotype and showed susceptibility to *Listeria monocytogenes* infection in *Tenebrio molitor* (Patnaik et al., 2015). Further, gene silencing of the vitellogenin gene resulted in poor adult development and egg production of *B. mori* (Lin et al., 2013). In the present study, our data indicate that the pupal hemolymph protein reduction might cause pupal deformity in the *B. mori*. Data presented here give a basic understanding and importance of the hemolymph proteins on growth, development and reproduction of insects.

Conclusion

The present study concludes that the oxidative stress on the hemolymph proteins by EBI exposure leads to developmental deformity in the pupae and obstruction in the adult emergence process of *B. mori*. Further molecular studies needed to reveal the effect of EBI on silkworm, *B. mori* is much warranted. This study conveys that application of <40 Gy of EBI will be much helpful in research to obtain mutant silkworm. As reported earlier, we support that EBI can be used as an effective tool for Insect Pest Management program.

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