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Picosecond UV laser induced morphological, biochemical and biological changes in *Bombyx mori*

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Background: In the light of various applications of UV laser in biological system, we have investigated the effect of picosecond UV laser radiation on silkworm *Bombyx mori*. ***Materials and Methods:*** The eggs of NB_4D_2 of different stages were exposed to pico second pulse laser at 355 nm from Nd:YAG laser for different durations. ***Results:*** Due to irradiation alterations in crescent larval body markings, pupae with transpositioned antennae, pseudo abdominal and caudal legs were produced from 2 and 8 hr old embryos irradiated for 30 and 50 seconds respectively. Moths devoid of antennae and underdeveloped legs were also produced from 16 and 8 hr old embryos irradiated for 60 and 50 seconds respectively. The morphological anomalies were found highest in picosecond (6.16%) compare to nanosecond (1%) irradiated embryos at 8 and 16 hrs respectively and it is duration dependent. SDS-PAGE analysis of embryo revealed the occurrence of a 41 kDa new protein and delayed utilization of yolk proteins in the irradiated embryos. The larval haemolymph protein profile also exhibited 24, 25 and 6.2 kDa new protein bands. Embryo hatching, larval weight and cocooning rate was significantly affected and declined as duration of irradiation increases. ***Conclusion:*** It is clear from the present study that morphological anomalies and distinct variations in egg and haemolymph proteins establish a strong evidence that UV picosecond laser not only cause damage on embryonic cells but also interfere in transcriptional factors encode for organogenesis and proteins. Thus present study envisage the use of UV laser irradiation as a potential tool in investigating the embryonic and postembryonic development and cross-linking between DNA and protein using silkworm *B. mori* as molecular model. **Iran. J. Radiat. Res., 2011; 9(2): 127-137**

Keywords: *Laser, UV, silkworm, radiation, Bombyx mori.*

INTRODUCTION

One of the fundamental aims of radiation biology is to study the biological effects that follow the initial energy deposition by irradiation. Radiations can produce a variety of damage in terms of mutations, chromosomal aberrations, genetic changes etc. Radiation studies have been extensively carried out in insects to bring a change in the genome of an organism utilizing both ionizing and non-ionizing rays. The silkworm, *Bombyx mori* is the only insect fully domesticated for commercial exploitation and has also long been used as an experimental material in various fields of biology [1-2]. In recent years, *B. mori* has been recognized as a molecular model insect not merely because of considerable importance in silk production but for the synthesis of recombinant proteins of pharmaceutical importance [3-4], regulation of tissue specific expression of silk protein genes in silk gland [5] and germ line transformation techniques [6-7]. The silkworm has also most genetically studied insect next to fruitfully with 500 mutants for wide variety of characters of which most of them were derived from ionizing radiations. Biological effectiveness of cosmic rays [8], magnetic energy [9], ultrasound [10], heavy ions [11-12] and laser [13-14] were also explored using *B. mori* as an appropriate model organism. In one of our previous reports, we have showed positive and negative affects of Helium-Neon laser on *B. mori* embryo [15]. Interestingly, laser facilities and its application in medical world have lavished as par excellence for various studies, like etiolo-

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gies, precise surgery and cell ablation etc. [16]. In addition, cross linking by short pulsed UV lasers is considered as potentially powerful tool to investigate DNA -protein interaction than any other chemical and enzymatic methods, because the number of photons required for covalent complex formation can be delivered very rapidly in nano, pico or even fentosecond intervals and the high energy of the pulses should result in efficient cross linking [17]. In the light of various applications of UV laser in biological system, we have investigated biomolecular changes and morphological anomalies at larva, pupa and adult stages induced by third harmonic UV laser radiation [18]. In continuation, here, we have followed an integrated approach to determine the cumulative impact of fourth harmonic UV laser (picosecond) radiation on hatching of embryo, morphological and biological traits, and egg and haemolymph protein profile of *B. mori*. Notably, published reports on the line of our interest do not exist although most of the UV laser studies confined to newly hatched larva [14].

MATERIALS AND METHODS

Experimental animal and irradiation. The cocoons of NB_4D_2 a bivoltine silkworm strain of *B. mori* (the popular and well acclimatized silkworm strain) used in the present study were procured from bivoltine seed cocoon market, Sirsi, Karnataka, India. The gravid moths were allowed to lay eggs in the laboratory. The laid eggs were collected every half an hour in a batch to achieve synchronization in embryonic development (the age of eggs was within half an hour and does not vary). The synchronized eggs were subjected for irradiation at different energy level. Cold acid treatment (HCl of 1.1 specific gravity at $25\pm2^{o}C$) was performed 20 hr after oviposition to prevent embryos from diapause. 2, 8, 16 and 24 hr old embryos from the time of oviposition were exposed to fourth harmonic laser (picosecond) pulses (355 nm from Nd: YAG laser, Continuum USA Model Locked and Q-switched of 35 psec) for different durations of 10, 20, 30, 40, 50 and 60 seconds. The repetition rate of the laser pulses was maintained at 10 Hz (hertz) with energy per pulse of 10 mJ (milli-joules). The pulse energy (10 mJ) was measured at a distance of 15 cm wherein the eggs were mounted. The diameter of the beam was 6 mm, which covers 20 to 22 eggs per exposure. The detail procedure for third harmonic laser pulses (nanosecond) was described elsewhere [18]. For each exposure, about 15 replications were maintained. After irradiation all the eggs were incubated at optimum environmental conditions until hatching. The percentage of hatching was determined as developmental index of an embryo.

The newly hatched larvae were reared along with control by following the standard procedure until spinning [19]. Morphological changes were analyzed during larval, pupal and adult period. The cocoons thus harvested were preserved under optimum environmental conditions for emergence of moths. All these cocoons were chopped at one end for detection of structural polymorphism at pupal stage and then they were allowed for emergence. On eclosion all the moths were observed for somatic mutations. The mutant moths were allowed for pairing followed by oviposition to determine the fertility. The rate of defect was calculated based on number of eggs irradiated with that of number of individuals exhibited morphological changes at larva, pupa and adult stages. A comparative analysis was made between pico and nano second laser and the data was subjected for statistical analysis using ANOVA.

Sodium Dodecyl Sulfate- Polyacrylamide Gel Eelectrophoresis (SDS-PAGE) of egg and haemolymph protein. Of the eggs exposed to UV-laser radiation at different developmental stages for varied duration an egg each from 8 and 16 hr after oviposition exposed for 40, 50 and 60 seconds was selected separately for protein analysis by

SDS-PAGE as described earlier [18] with suitable modification [20].

Biological parameters. The biological parameter refers to hatching, larval weight and cocooning percentage were reported. The average hatching percentage (mean of all replications of each irradiation group) was calculated after complete hatching of irradiated and control groups of eggs. For larval weight, on day 6 of fifth instar, 10 larvae were randomly selected from each irradiated group of each replication and average of three replications was recorded. Cocooning percentage was calculated considering number of cocoons spun by total number of larvae irradiated and control. All the data were subjected to Statistical (ANOVA) analysis.

RESULTS

Morphological anomalies

Exposure of silkworm embryos at varied embryonic stages to high-energy UV laser (Picosecond) exhibited various anomalies at larval, pupal and adult stages. The crescent marking had split and exhibit tetrad pattern in the larvae derived from embryos irradiated at 16 hr for 30 seconds (figure 1A). Whereas, some larva had either one crescent marking or one star marking or partial expression or none of them (figures not shown). The pupae with adhenate antennal buds fused distally and transpositioned in opposite direction were derived from 2 hr old embryos irradiated for 30 seconds (figure. 1B). On ecloson, the moth had fused antennae with undifferentiated flagellum and sparsely spaced annular branches (figure 1C). Interestingly, the female moth paired with normal moth laid fertile eggs. Some of the resultant F_1 larvae were of mosaic type and survived until third instar and other offspring's were found normal.

A pair of vestigial legs was noticed distinctly on third, fourth, fifth and sixth abdominal segments in the pupae obtained from 8 hr embryos irradiated for 50 seconds (figure 1D). Some of these pupae were dead at late pupal stage and few emerged as normal adult. In addition, mosaic pupae with chocolate body colour were derived from embryos irradiated at 8 hr for 50 seconds, which were dead at late pupal stage (figure 1E). Notably, while one of the antennae completely disappear the other was found normal in the moth derived from irradiated embryo at 16 hr for 60 seconds (figure 1F). Interestingly, one of the meta-thoracic legs in the moth derived from 8 hr old embryo (50 seconds) was severely affected, while as tibia, tarsus and pre-tarsus completely degenerated, the coxa, trochanter and femur (figure 1G) remain normal. Moths with short, deformed and crumpled wings were uncommon (figures not shown) and exhibited normal behavior.

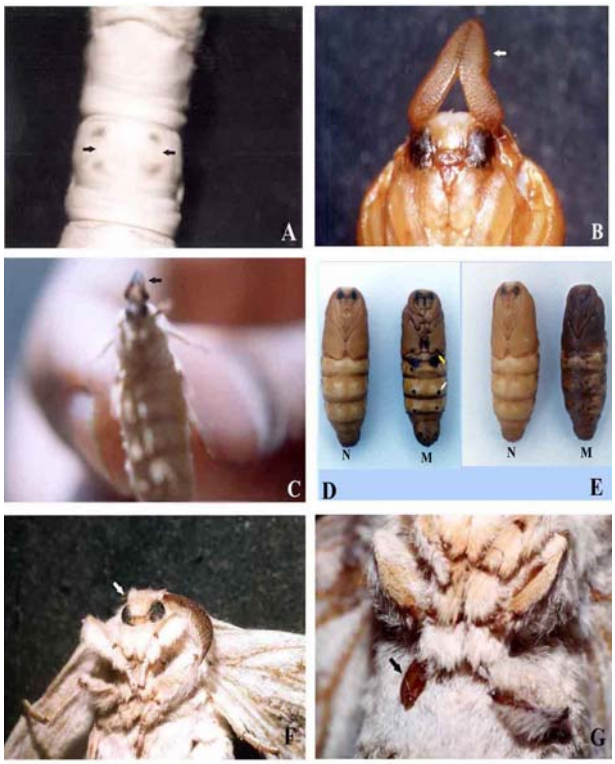

Figure 1. *Bombyx mori* larva explicit split crescent markings as two pairs - arrow (A-1.8x). Pupa with agglutinated antennal buds, arrow indicates fusion of antennal bud (B-5x). Metamorphosed adult with persisted transpositioned and fused antennae (Black arrow) (C-1.2x). Mutant pupae (M) with pseudo abdominal and caudal legs (White arrow) and normal (N) pupa (D-1.3x). Mosaic pupae with chocolate colour (E-1.4x). Moth with one-missed antennae (white arrow) and the other normal. (F-8x). Moth with deformed metathorasic leg (Black arrow) (G-11x).

Frequency of morphological variations

Irradiation of silkworm embryos at its different developmental stages exhibited varied morphological defects at larval, pupal and adult stages which were persisted either at any one of the stages (discontinuous) or continued in proceeding stages (continuous). The highest morphological variations observed in pico and nanosecond was 9.51 (50 seconds) and 1.22 percent (150 seconds) respectively (figure 2). Comparatively, 8 hr old embryos were found to be very sensitive than 2 and 16 hr old embryos, wherein 6.16 and 1.00 percent somatic mutation was recorded in pico and nanosecond lasers respectively (figure 3). The frequency of structural variations increases as duration of irradiation increase up to 50 seconds and thereafter it decreases in picosecond, whereas it differs in nanosecond (figure 2).

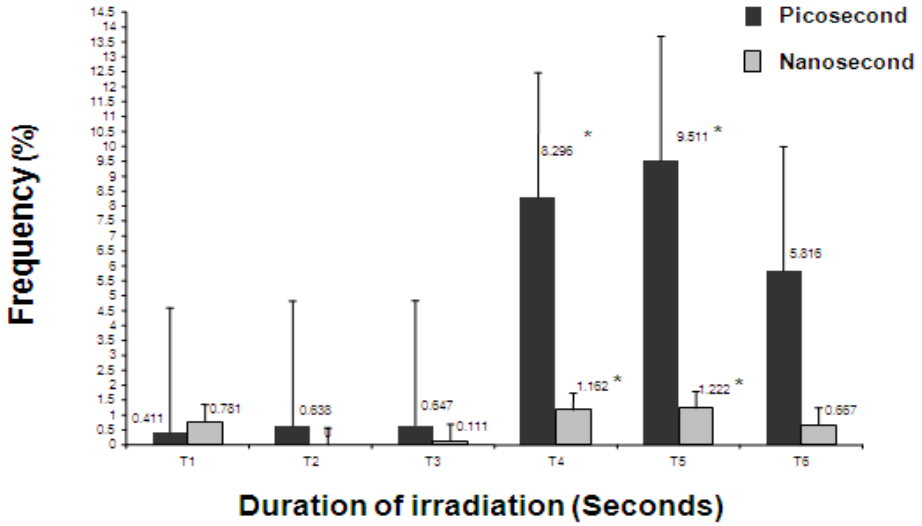

Figure 2. Effect of different duration of nano and picosecond UV lasers on *Bombyx mori* embryo as revealed by morphological changes during post embryonic development. T1: treatment-1 refers to irradiation of silkworm embryos for 10 seconds in pico and 30 seconds in nanosecond UV laser (T1-p10/n30);T2-p20/n60; T3-p30/n90; T4-p40/n120; T5-p50/n150; T6-p60/n180). Numerical on bars are actual readings and lines on bars are standard error bars. * Significant at 0.5; **significant at 0.1.

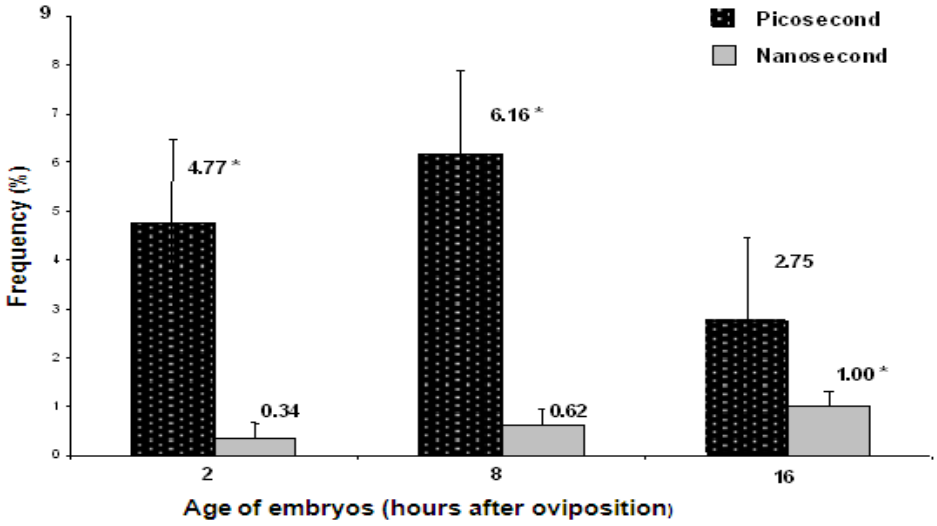

Figure 3. The total morphological anomalies during larval, pupal and adult stages as induced by nano and picosecond UV lasers in accordance with age of embryo in *Bombyx mori*. Numerical on bars are actual readings and lines on bars are standard error bars. *significant at 0.5; **significant at 0.1.

Egg and haemolymph protein

A comparative analysis of embryonic protein derived from irradiated and control population revealed qualitative and quantitative variations. No major changes were noticed in the protein profile until eight day but considerable amount of yolk proteins ascribable to vitellin-H (Vtn-H, 178 kDa), egg specific protein (ESP- 66 kDa), vitellin-L (Vtn-L, 43 kDa) and 30 kDa proteins remain distinct and discrete in irradiated groups (figures not shown). Notably, on ninth day, the protein profile of embryo irradiated at 8 and 16 hr for 40, 50 and 60 seconds revealed delayed utilization of Vtn-H (178 kDa), ESP (66 kDa), and Vtn-L (43 kDa), where as in control all these yolk proteins were disappeared, although all these populations belonging into the same age group. Concurrently, protein bands with molecular mass of 38 and 45 kDa appeared in the embryo irradiated at 16 hr for 50 (figure 4A, lane 7) and 60 (figure 4A, lane 8) seconds were similar to that of control. But appearance of 41 kDa protein in the irradiated group than in control of same age group of embryo was significant (figure 4A, lanes 7&8).

SDS-PAGE analysis of haemolymph protein of fifth instar larva derived from irradiated embryo revealed significant changes (Fig.4B). A major change with the appearance of two new proteins of 25, and 26 kDa was noticed in the haemolymph of sixth day larva derived from 2 hr old embryo irradiated for 40 seconds (Fig.4B, lane 2). On eight day, a low molecular weight polypeptide of 6.2 kDa was noticed in the sample derived from 2 hr old embryo irradiated for 40 seconds (figure 4B, lane 4).

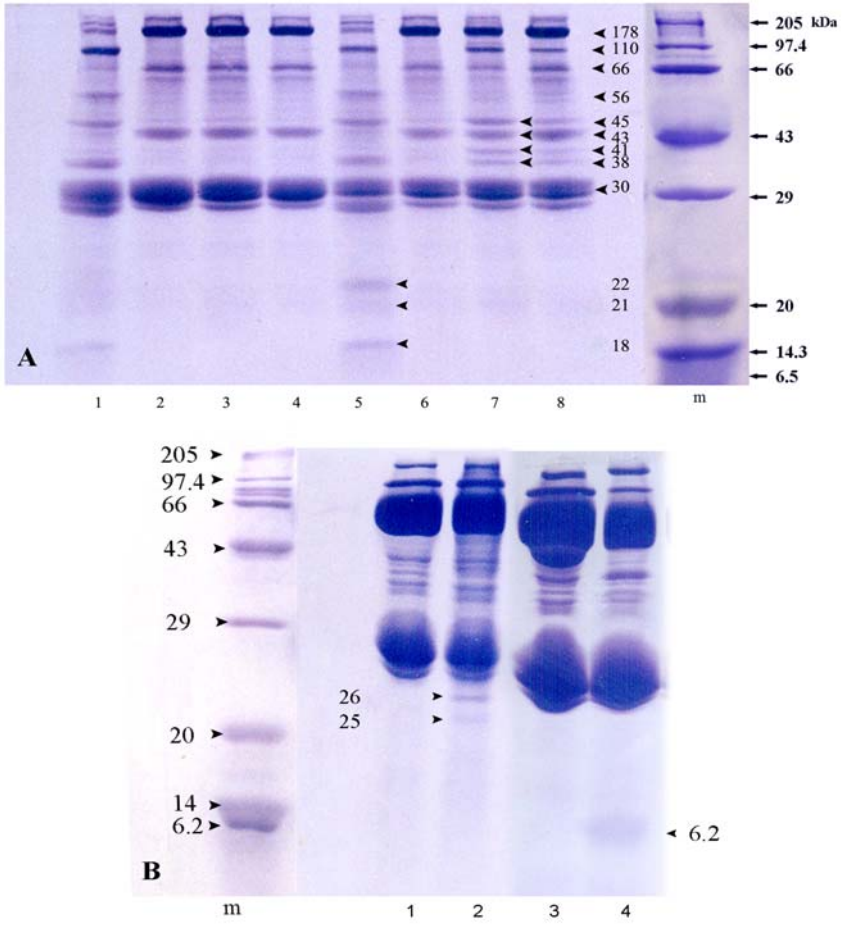

Figure 4. A) *Bombyx mori* embryonic protein profile. Lanes 1 to 4 for 8 hr embryos. Lanes 5 to 8 for 16 hr embryos. Lanes 1 & 5-control. Lanes 2 & 6 –40 seconds. Lanes 3 & 7 -50 seconds. Lanes 4 & 8 -60 seconds; m-molecular marker. 9th day embryonic protein profile with persisted major yolk proteins in lanes 2,3,4,6,7 and 8; in addition 41 and 38 kDa protein bands in lanes 7 and 8 is significant. Appearance of 110,56,45,22,21 and 18 kDa proteins in lanes 1 and 5 depicts the changes in protein profile during normal embryo development. **B)** *Bombyx mori* larval haemolymph protein profile. Lanes 1 and 3 - control of 6th and 8th day for 8 hr embryos; m-molecular marker. 6th day larval haemolymph protein profile with new proteins of 26 and 25 kDa in lane 2. 8th day larval haemolymph protein exhibit new protein of 6.2 kDa in lane 4.

Biological traits

Hatching: The percent of hatching of embryos was significantly affected due to UV laser irradiation in the groups examined. The hatching of embryos was decreased to 60.66% from the eggs irradiated for 2 hr over 60 seconds, which is 31 percent less compare to control (88.05%). Where as, the highest hatching of 84.8 (4% decrease over control) was noticed in the group consisted 8 hr old embryos exposed to UV laser for 10 seconds. Interestingly, a gradual decline in hatching of embryos was obvious in the entire irradiated group irrespective age of the embryos as duration of exposure increases (figure 5).

Larval development: The day 6 of fifth instar larval weight was considerably decreased in the entire irradiated population compare to non-irradiated group (figure 6). The larval weight in the control group was 3.148 g, where as in irradiated groups the maximum larval weight of 3.078 g (2% decreases over control) was recorded from 16 hr old embryos irradiated for 20 seconds. Concurrently, a least larval weight of 2.319 g (26% decreases over control) was recorded from the 8 hr old embryos irradiated for 60 seconds. The larval weight was consistently decreased as the exposure period increase (figure 6).

Cocooning*:* The cocooning percentage was significantly decreased in the groups exposed to UV laser irradiation. While, non-irradiated silkworm larvae exhibit 92.55% (highest) of cocooning, concomitantly, the silkworm larvae derived from 8 hr old embryos irradiated for 60 seconds showed 62.49% (lowest) of cocooning, which is 33 percent decrease over control. As duration of irradiation period increase the cocooning rate was also decreased (figure 7).

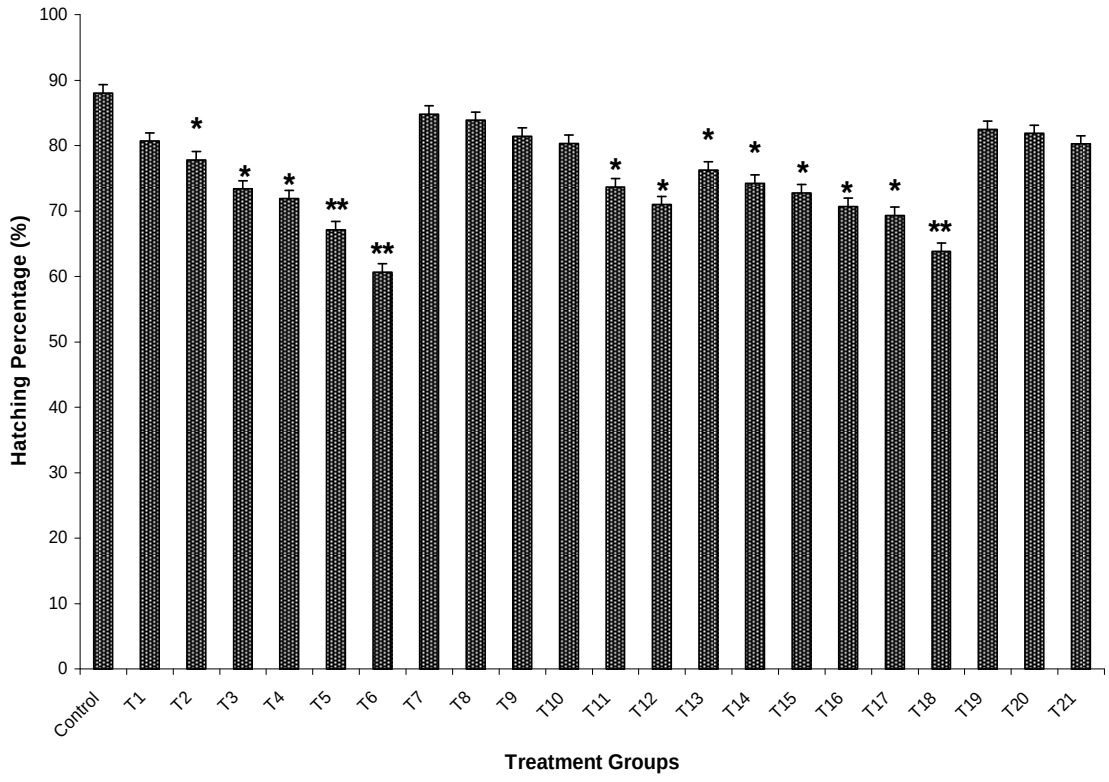

Figure 5. Effect of fourth harmonic UV laser on hatching of embryo of *Bombyx mori*. Letter T represents treatment group; T1 to T6 (10,20,30,40,50&60 sec) - irradiation of 2 hr old embryo, T7 to T12 (10,20,30,40,50&60 sec)-irradiation of 8 hr old embryos, T13 to T18 (10,20,30,40,50&60 sec)- irradiation of 16 hr old embryos, T19-T21 (10,20&30 sec) – irradiation of 24 hr old embryos.

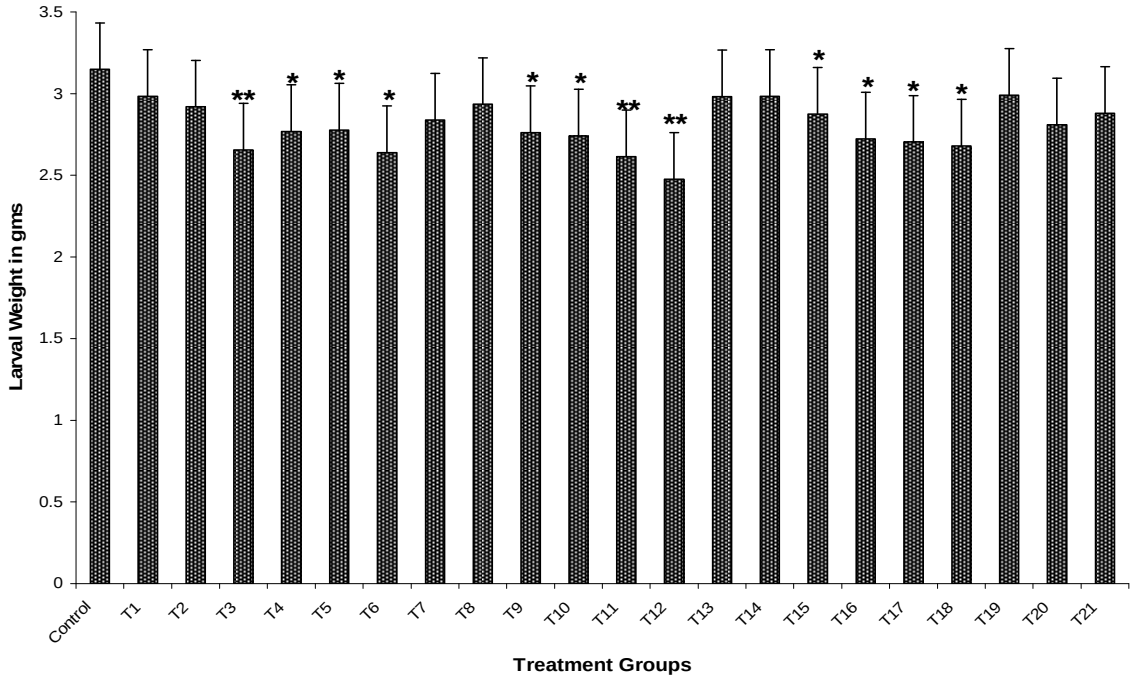

Figure 6. Day 6, fifth instar silkworm *Bombyx mori* larval weight as influenced by fourth harmonic UV laser irradiation on different embryonic stages. Letter T represents treatment group; T1 to T6 (10,20,30,40,50&60 sec) - irradiation of 2 hr old embryo, T7 to T12 (10,20,30,40,50&60 sec)-irradiation of 8 hr old embryos, T13 to T18 (10,20,30,40,50&60 sec)- irradiation of 16 hr old embryos, T19-T21 (10,20&30 sec) – irradiation of 24 hr old embryos.

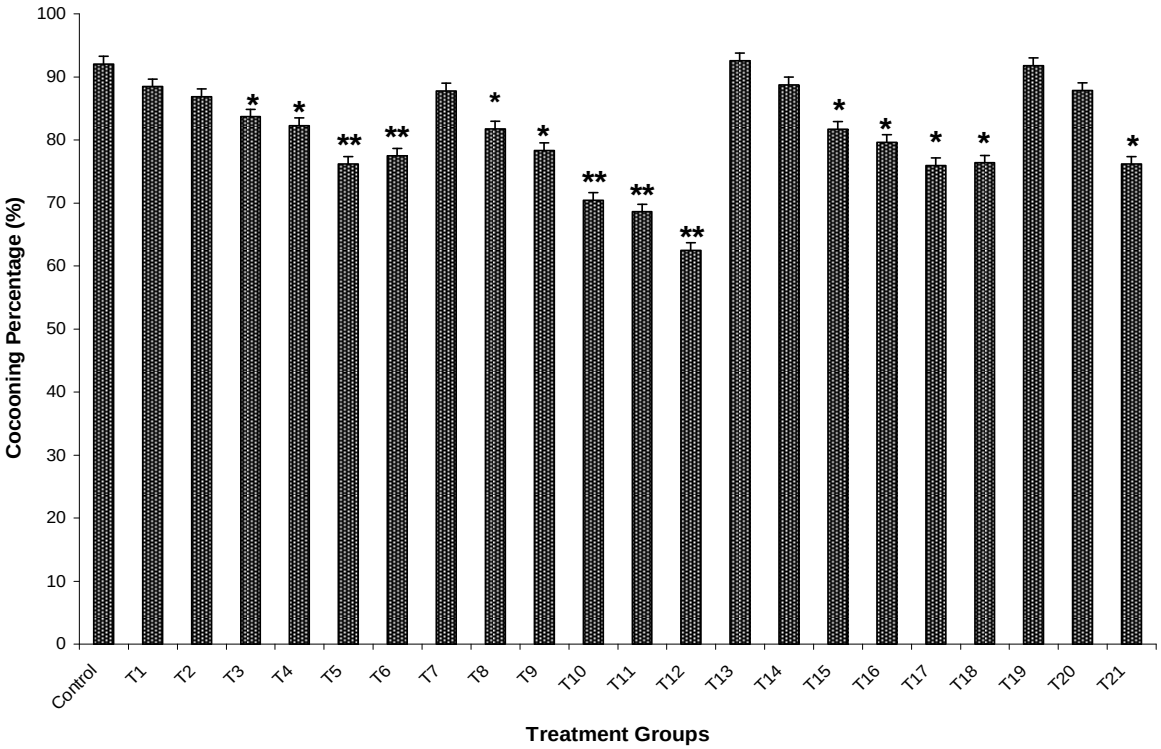

Figure 7. The cocooning percentage of silkworm *Bombyx mori* as influenced by fourth harmonic UV laser irradiation on different embryonic stages. Letter T represents treatment group; T1 to T6 (10,20,30,40,50&60 sec) - irradiation of 2 hr old embryo, T7 to T12 (10,20,30,40,50&60 sec)-irradiation of 8 hr old embryos, T13 to T18 (10,20,30,40,50&60 sec)- irradiation of 16 hr old embryos, T19-T21 (10,20&30 sec) – irradiation of 24 hr old embryos.

DISCUSSION

Morphological traits

To study the molecular basis of biological effectiveness of high-energy UV laser (picosecond), the whole embryo of *Bombyx mori* strain NB_4D_2 was used, because, radiation of silkworm eggs is an efficient strategy for generating additional mutations [21] and it was strongly evidenced with our earlier studies using third harmonic UV laser [18]. Irradiation of whole embryo at its different stages to fourth harmonic UV laser also exhibited significant morphological variations at larval, pupal and adult stages as it was observed in third harmonic UV laser but type and frequency varies. Basically, the silkworm strain NB_4D_2 posses p or p^1 degree of expression but larval crescent and star markings observed in the present study are of p^2 degree due to UV laser irradiation, which confirm the earlier observation [18] as because p^{-S} (p allelic gene) is responsible for the fundamental body marking pattern [22].

The characteristic transposition and distally fused antennae in moth, being unique feature of the study, persisted through metamorphosis of pupae (it was difficult to see in larvae as antenna were smaller in size) clearly indicate the change in gene expression or embryonic antennal cells once damaged due to high energy UV laser irradiation did not experience massive cell arrangements during metamorphosis. Further, this trait endorsed our earlier findings where in simple and complex fusion of abdominal segments induced by nanosecond UV laser persisted through larva-pupa-adult [18]. These distinct changes induced at embryonic stages are due to impact of UV laser both in nano and picosecond E-allelic groups E^{Ca}, E^{D}, and E^{KP} that determine differentiation of body segments in early embryonic stages [1]. Normally, thoracic legs of larva transformed as adult legs, while abdominal legs sloughed off during metamorphosis. Interestingly, rudimentary abdominal and caudal legs were noticed in pupal population derived from picosecond laser irradiated embryos, which, we could not noticed in larvae as reported by [13] but impact of UV laser on legs as that of antennae of silk moth is obvious. Recently, the serial analysis of gene expression revealed radiation induced changes in 57 genes with altered transcription levels in *B. mori* [21]. Thus, distinct changes induced at embryonic stage, which in turn appeared at larval, pupal and adult stages either as continuous or discontinuous not only depicts the impact of picosecond and nanosecond UV laser on hereditary (genetic) traits of *B. mori* also substantiate our earlier findings [18]. However, further investigation on molecular mechanism underlies that includes chromosomal aberrations, DNA damage etc. is offered.

Frequency of morphological anomalies

The present experiments explicit the age, dose and time dependent effectiveness of UV laser (nano and pico seconds) on silkworm embryo. Here, we have evaluated the rate of morphological variations that occur at larva, pupa, and adult stages for the first time as UV laser induced defects were reported only from newly hatched larvae [13-14]. Although, the frequency of morphological anomalies increases as the energy level increases from nanosecond (1.22%) to picoseconds (9.51%) but type of morphological defect (somatic mutations) and protein profile varies. For example, asymmetrical and complex fusion of abdominal segments observed in larvae, pupae and adults derived from the embryos irradiated with nanosecond [18], where as transpositioned antennae in pupae and adults was recorded from the embryos irradiated with picosecond UV laser. The alteration in larval markings was uncommon in both third and fourth harmonic irradiation. Recently, of the 710 irradiated specimens using localized UV laser irradiation, 452 showed two normal silk glands, 27 showed one normal and one deformed gland, 21 showed one normal gland only, and 210 showed no silk gland

and concluded that the site of irradiation that caused deletion of a silk gland were located with in the area of irradiation [13]. Among different stages of embryonic development 8 and 16 hr old embryos irradiated at different pulses yielded highest malformations. This difference could be due to number of cells (cleavage nuclei) exposed to UV laser at the time of irradiation which are supposed to be involved in cellularization during embryonic development.

Biochemical traits

Further, we have analyzed the protein profile of irradiated embryos with the control through SDS-PAGE by comparing with that of protein map of *B. mori* [18]. It is inferred and confirmed from the present study that high energy level of UV laser also altered egg protein profile with appearance of new protein band having molecular weight 41 kDa and much delayed utilization of yolk proteins as that of nanosecond UV laser irradiation [18]. Basically, Vtn-H and L, ESP and 30k proteins are the major yolk proteins of silkworm egg and have specialized function in the physiological events during embryonic life [23]. These yolk proteins are degraded at different stages during embryogenesis by different proteases [24-25] as has observed in the control population of the present study and their activities are assumed to be regulated by interaction with the protease inhibitors which are stored in the yolk [26]. 30k-protease and 24-protease disappeared at the time of larval hatching [23, 25] but degradation of 30k proteins is catalysed by proteases that are different from the 30k-protease and 24-protease [27-28]. Surprisingly, on eight and ninth day, the yolk proteins ascribable to Vtn-H (178 kDa), ESP (66 kDa), Vtn-L (43 kDa) and 30 kDa proteins remain unchanged in irradiated groups where as in control all these yolk proteins were disappeared. Hence, delayed and differential degradation of these major yolk proteins along with other proteins may due to UV laser induced modification in interaction of the protease inhibitors which are stored in the yolk with the proteases that are essentially required for embryonic life.

Apparently 41 kDa protein band consistently observed both in present (figure 2A, lanes 7&8) and earlier [18] investigations but their biological significance is enigmatic. However, it might have some biological significance, as the early consumption of 6G1-30K-1 and the higher accumulation of 6G1-30K-3 and 6G1-30K-4 is highly caused by the destruction of physiological balance in normal embryonic development, which may lead to lower hatchability [29]. The N-terminal amino acid sequence of this protein might give its structural and functional significance. Further, we have also noticed slow embryonic development as evidenced by delay in utilization of yolk proteins due to laser irradiation as noticed by Nirmala *et al.* [30] using UV irradiation.

A new protein with molecular weight 6.2 kDa found only in the haemolymph of the eight day-fifth instar UV laser irradiated larvae by SDS-PAGE which is different from that of storage protein could be attributed to phenotypic variation observed in the present study. Similar to larval serum protein [31], molecular characterization of new protein noticed might enlighten the functional and biological significance in *B. mori*.

Thus biomolecular changes observed in *B. mori* for the first time establishes the strong molecular phenomenon underlies in structural variations as induced by UV laser and agrees with the opinion of Kiguchi *et al.* [12] that UV laser affects not only nucleic acids but also proteins and membranes, while the primary targets of heavy ions are nuclei or DNA.

Biological Traits

It is evident from the earlier studies that sensitivity to radiation is very high in early embryonic stages, such as blastoderm formation and germ band stages [1]. Hence, in the present investigation, we have considered these stages to determine the

effectiveness of the fourth harmonic UV laser on hatching, larval growth and economic parameters. The hatching of embryos declined in all the embryonic stages (2, 8, 16 and 24 hr) examined in accordance with that of increased period of exposure to UV laser as has been reported [30-33] that embryonic mortality is dose dependent. The embryonic mortality is due to delayed utilization and degradation of embryonic proteins (figure 4), alterations in the expression of proteases and also might be inhibition of mitosis [34] in the irradiated eggs. Further, the high embryonic mortality in 2 hr irradiated embryos might be due to damaging of egg nuclei by UV laser irradiation [13]. Notably, the impact of UV laser irradiation is not only confined to embryonic stage also reflected on larval growth and development. As a consequence the weight of the larval mass was significantly decreased in all irradiated groups compare to that of unirradiated larval group. Even, radiation studies in silkworm *B. mori* attain a special significance due to its economic value among other insects. UV laser irradiated population revealed negative effect on economic traits, wherein decreased cocooning percentage. This inference is also supported by Shigematsu and Takeshita [35-36] studies that the gamma irradiation on the formation of fibroin was more conspicuous than on sericin due to decreased synthesis mRNA in the posterior division of the silkgland, which intern limits the rate of template formation for the fibroin synthesis.

In conclusion, it is clear from the present and earlier [18] investigations that structural polymorphism (somatic mutations) induced by UV laser in support of distinct variations in egg and haemolymph protein profile although an unique findings also establish a strong evidence that UV laser (picosecond) not only cause damage on embryonic cells but interfere in some transcriptional factors encode for organogenesis and egg, haemolymph and silk proteins. The recent findings support our view that RNA fragments may be useful as photoprotective agent with *in vivo* effects comparable to DNA repair enzymes [37]. Thus, the use of UV laser irradiation with different energy level could be a potential tool to uncover the critical transcriptomes involved in embryonic and postembryonic development and cross-linking between DNA and protein [17] using silkworm *B. mori* as molecular model candidate.

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