CHAPTER - 2

EFFECT OF SPERMIDINE ON
GROWTH, METABOLIC STATUS, AND
OXIDATIVE STRESS DURING SILK
GLAND DEVELOPMENT
1 INTRODUCTION

1.1 PAs in growth

PAs are important for the cell growth and their depletion caused growth inhibition. PAs can modulate DNA-protein interactions, gene expression, translation and enzymatic activities (Kusano et al., 2008, D’Agostino et al., 2005, Ouameur et al., 2004). The potential mechanism of PAs supporting mammalian cell proliferation was studied in mouse fibroblasts and HEK-293T (human embryonic kidney cells) cell lines (Guy Landau et al., 2010). Regenerating tissues require PA synthesis. Spd is needed for the proliferation of hair follicle and to promote human hair shaft elongation (Ramot et al., 2011). The effect of Put, Spd, and Spm on the expression of keratin 1 protein was studied using human keratinocytes. Results showed Spd and Spm treatment up-regulated the expression of keratin 1 protein. PAs were proven to be nutrient agent to maintain healthy reproductive system. In male reproductive system the expression of PAs correlates with stages of spermatogenesis (Lefevre et al., 2011). PAs also play role in formation of placenta and implantation of embryo. Spd and Spm feeding to B. mori 5th instar larvae increased the egg production (Danti et al., 2016).

2 PAs and longevity

PAs remove reactive oxygen species. They protect lipids, DNA and protein from oxidative damage (Chattopadhya et al., 2006). Feeding of PA caused longevity in Callosobruchu maculates F (Garg and Mahajan 1993). In human cell lines, PAs increased resistance to H2O2 and decreased oxidative damage (Eisenberg et al., 2009). Spd prolonged the life span of yeast, nematodes and fruit flies. In mice, they reduced age-related protein damage caused by oxidants (Madeo et al., 2010). Mice fed with high PAs showed higher survival rate (Soda et al., 2009). The present study was carried out to check PAs effect on metabolic status and antioxidant potential of silk glands during 5th instar larval and pre-pupal developmental period of silkworm strain, CSR 2 × CSR 4.
2 METHODS

2.1 Measurement of Silk Gland Weight

Larvae were selected randomly in both the groups and scarified for gland weights. Gland weights were measured in both control and Spd treated groups during the 5th instar larval silk gland developmental period (day 1, day 3, day 5, day 7, and day 9). The images of isolated glands of both control and treated group were represented in appendix fig. 2.

2 Cell Viability Assay

MTT assay was performed with silk glands of B. mori based on earlier protocol (Nath et al. 2005) to check cell viability. Five randomly selected larvae of both control and treated group were dissected and silk glands were isolated. The glands were weighed and kept in tubes containing of insect ringer solution (900 µl) and MTT (100 µl of 5 mg / ml). Tubes were incubated for one hour in dark. MTT solution was discarded after 1 hour of incubation. To the glands 50 % (v / v, 1 ml) tween-80 solution was added and incubated for 24 hours for extraction. After incubation, the formazan product was collected and the total volume was made to 3 ml. Samples were vortexed and absorbance readings were taken using colorimeter at 570 nm (Elico, CL - 157, India).

2 Antioxidant Assays

Silkworms of both treated and control worms were dissected in 1X PBS. Five worms were randomly selected from each group, and the assays were done in triplicate.

1 DPPH Assay

Standard published protocol was used for DPPH assay (Zhao et al. 2006). Silk glands of treated and control were homogenized in 1ml of ice cooled, 0.1 M phosphate buffer. After homogenization, samples were centrifuged at 4°C for 10 minutes at 10,000 rpm. The collected supernatant was used for analysis. DPPH solution was prepared by dissolving DPPH crystals in 100 % ethanol and stored at 4°C overnight and protected from light. The solution is prepared one day before the use. DPPH (is a stable free radical molecule, which is dark purple in colour. 200 µl of supernatant was taken from the gland homogenate. To the homogenate (800 µl), 0.1 mM DPPH solution was added and kept in dark for 30 minutes (As). Ethanol was taken as negative control and ethanol with DPPH as positive control was taken (Ac). The O.D readings were taken at 520 nm using spectrophotometer. Auto zero
calibration was done with negative control. % Inhibition of free radicals was calculated using the given formula.

\[ \% \text{ Inhibition} = \frac{(Ac-As)}{Ac} \times 100 \]

2.2.2 FRAP Assay

Standard published protocol was used for Ferric reducing antioxidant power (FRAP) assay (Wong et al., 2006) with slight modification. This assay is used to measure the total antioxidant ability which involves pro-oxidant or an oxidizing substrate. Silk glands were homogenized in chilled 0.1 M phosphate buffer and supernatant was collected. 200 µl of sample was added to FRAP reagent (3 ml). All the solutions were prepared separately and mixed in the ratio 10:1:1. Tubes were incubated for 30 minutes at 37 °C in dark. The reaction is mainly based on the reduction of ferric ion to ferrous ion present in TPTZ. This reduction produces blue colour. The absorbance readings were taken at 593 nm using spectrophotometer. Reaction mixture devoid of sample was taken as blank. The variation in O.D was calculated as given below.

\[ \Delta \text{Absorbance} = \text{Sample absorbance} - \text{Blank absorbance} \]

3 SOD Assay

Standard published protocol was used for superoxide dismutase assay (Beauchamp and Fridovich 1971). Silk glands of both control and treated groups were homogenized in ice cooled 0.1 M phosphate buffer and centrifuged at 10,000 rpm for 10 minutes at 4 °C. 10 µl of homogenate was added to 3 ml of reagent mixture (As). The reaction mixture without extract was taken as control (Ac). The tubes along with reaction mixture and sample were exposed to 400 W bulbs. % Inhibition was calculated as given below.

\[ \% \text{Inhibition} = \frac{(Ac-As)}{Ac} \times 100 \]

4 CAT Assay

Standard published protocol was used for catalase activity assay (Madhusudhanm et al., 2012). Silk glands were homogenized in cold 0.1 M phosphate buffer and centrifuged at 10,000 rpm for 30 minutes. The reaction mixture containing 1 ml of 0.1 M phosphate buffer, pH 7.0 along with crude extract was taken into sample cuvette and 10 µl of H$_2$O$_2$ substrate was added. Reaction mixture was then immediately scanned at 240 nm every 12 sec for 2
Decomposition of hydrogen peroxide was measured as decrease in absorbance at 240 nm. Standard catalase activity was performed as described earlier. The catalase activity was expressed as

\[
\text{Activity (U) / min}
\]

3 Statistical analysis of the data

The experiments were replicated and all the experimental data was subjected to statistical analysis by following t-test; two sample assuming equal variances \( (P (T<= t) \text{ one-tail} = 0.05) \) and by two-way ANOVA with time and treatment as fixed effects.

3 RESULTS

3.1.1 Effect of Spd on Silk Gland Weight

Silk gland weights were recorded on every alternate day of 5th instar during the larval developmental period (Fig 2). An increase in the gland weights was observed from day 1 to day 7. Sudden decrease in silk gland weights was observed on day 9. The sudden decrease of gland weight on day 9 could be due to non-feeding of larvae. They enter into spinning stage passing wandering stage. During this stage the stored protein in the silk gland excretes out as a silk fiber and could have lead to decrease in the silk gland weights. Spd feeding to the larvae increased the gland weights when compared to control. The result correlated with the increase in the body weights after Spd feeding. Significant increase in the gland weights on day 3, 5 and 9 were observed. Two way ANOVA analysis with replicates of fixed effect of time and treatment showed time and treatment showed positive effect on gland weight \( (p < 0.001) \). The analysis showed the interaction between development and treatment had no effect \( (p = 0.152) \).

The Spd 50 µM showed positive effect on silk gland weights. The silk gland weight remained significantly higher than control group on day 9.
Effect of Spd on silk gland weight during development

Fig 1: Effect of Spd on silk gland weights during larval and pre-pupal development. X-axis represents different days of treatment and Y-axis represents silk gland weights in (gm.). Mean taken from three different treatments were plotted along with error bars to represent data variability (n = 5). *p < 0.05 **p < 0.001.

2 Effect of Spd on Metabolic Status of Silk Gland

The metabolic activity of control and Spd 50 µM treated silk gland were checked on every alternate day during the 5th instar larval developmental period (Fig 3). Spd was shown to increase the longevity in many organisms. The metabolic activity of silk gland increased from day 1 to day 7 and decreased on day 9 in both control and treated groups. The metabolic activity correlated with the silk gland weights. Significant increase in metabolic activity was observed on day 3, day 5, and day 9. Two way ANOVA analysis showed that time and treatment had positive effect on metabolic activity (p < 0.001), but the interaction between time with treatment showed no significant effect (p = 0.599).

Spd feeding to 5th instar larvae showed increased metabolic activity of silk glands in larval and pre-pupal development.
Effect of Spd on Silk Gland Cell Viability during 5th instar Development

Fig 2: Effect of Spd 50 µM feeding on cell viability of silk glands. MTT assay was performed with silk glands of control and treated groups during 5th instar larval (day 1-day 7) and pre-pupal developmental period (day 9). Average O.D (n = 5) with standard errors was plotted. X-axis represents different days of development and Y-axis represents absorbance in O.D units at 570 nm. *p = 0.05, **p = 0.01 (student t-test).

2 Effect of Spd on Antioxidant Assays

3.2.1 DPPH Assay

The free radical scavenging assay was performed for silk gland homogenates on every alternate day from day 1 to day 7 during the larval developmental period and day 9 of pre-pupal stage (Fig 4). To maintain the cell viability free radical scavengers are very important. Result showed an increase in the % inhibition of free radicals from day 1 to day 7 and decreased on day 9 of pre-pupal stage in both control and treated groups. Spd treated group showed more inhibition than control group. Significant (p = 0.020) increase in the % inhibition was observed on day 9 of pre-pupal stage. Two way ANOVA analyses were performed with fixed effects of time and treatment. The results showed both time, treatment and time vs treatment had positive effect (p < 0.05).
Effect of Spd on Free Radical Scavenging of Silk Gland

Fig 3: DPPH assay of silk gland homogenates during 5\textsuperscript{th} instar larval (day 1 to day 7) and pre-pupal (day 9) development stage of control and treated groups. X-axis represents different days of development and Y-axis represents the % Inhibition of free radicals (n = 5). *$ p < 0.05$.

3.2.2 FRAP Assay

Total antioxidant capacity of silk gland homogenates were analysed for both control and treated groups during every alternate day of larval developmental period and pre-pupal stage by spectrophotometer (Fig 5). The results showed increase in total antioxidant capacity of silk gland homogenates from day 1 to day 9 in both control and treated groups. Significant increase was observed on day 3 in treated group ($ p = 0.007$). Two way ANOVA analyses showed no significant effect influence of time. The treatment showed highly significant effect ($ p < 0.001$). The interaction between time with treatment had no significant effect ($ p = 0.759$).

Effect of Spd on Total Antioxidant Potential of Silk Gland

Fig 4: FRAP assay of silk gland homogenates during 5\textsuperscript{th} instar larval (day 1 – day 7) and pre-pupal (day 9) stages of control and treated groups (n = 5). Change in absorbance values along with standard errors were plotted. X-axis represents the different days of development and Y-axis represents change in Absorbance ($\Delta$ absorbance) values at 593 nm. *$ p < 0.01$.

3 SOD Assay
Super oxide dismutase enzyme (SOD) activity plays an important role in relieving oxidative stress. SOD activity was measured with silk gland homogenates on every alternate day with both control and treated groups (Fig 6). The percentage of inhibition was calculated from the absorbance values. The activity increased from day 1 to day 7 during the 5th instar silk gland developmental period and decreased on day 9 of pre-pupal stage in both the groups. Compared to control group, treated group showed more enzyme activity. Significant increase was observed only on day 7 in the treated group. Two way ANOVA analyses showed positive effect of time and treatment on SOD activity. The effect of time is significant ($p = 0.04$). Treatment also showed highly significance difference in SOD activity ($p < 0.001$). The interaction with time vs treatment did not show any significant effect ($p > 0.05$).

**Effect of Spd on Superoxide Dismutase activity of Silk Glands**

*  

**Fig 5**: SOD enzyme activity of silk glands homogenates during 5th instar larval (day 1 – day 7) and pre-pupal (day 9) stage of control and treated groups ($n = 5$). Mean taken from three different treatments were plotted along with error bars to represent data variability. X-axis represents different days of development and Y-axis represents % Inhibition. *$p < 0.05$.

4 **CAT Assay**

In addition to SOD activity, CAT assay was performed with silk gland homogenates on every alternate day during different days of silk gland development (Fig 7). Increase in the activity was found in treated group. But significant increase was observed on day 7 ($p < 0.05$). The ANOVA analysis showed that the treatment had significant effect ($p = 0.030$) on CAT activity. Time and interaction between time vs treatments showed no significant effect on the enzyme activity ($p > 0.05$).

**Effect of Spd on Catalase activity of Silk Glands**
**CONCLUSION**

Antioxidant potential of the silk gland homogenates increased during 5th instar larval stage and found to be decreased during the pre-pupal stage. This could be due to increase in age as the glands prepare for autophagy after day 7 or due to decreased metabolic activity as larvae go to non-feeding, wandering stage on day 9. The decreased metabolic activity of silk glands and antioxidant potential could have resulted in increased oxidative damage to protein and DNA and helped in its degradation. Silk glands of Spd treated larvae showed increased metabolic activity and antioxidant potential when compared to the control silk glands. Thus, the increase in antioxidant potential observed in the treated glands could be due to the direct action of Spd as a free radical scavenger as reported earlier.

It was concluded from the study that Spd 50 μM feeding to *B. mori* larvae increased metabolic activity and antioxidant potential of silk glands during 5th instar larval and pre-pupal developmental stages.