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Histological assessment of toxicity impact of various dyes on midgut of fifth instar of mulberry silkworm, *bombyx mori*

B Nazeer Mohamed[1], R Kebaraj[1], P Mohideen Askar Nawas[1], Athmanathan Balasundaram[1], H F Seyed Mafiya Haniff[2]
[1] Department of Zoology, Thanthai Periyar Government Arts & Science College, (Affiliated to Bharathidasan University) Tiruchirappalli, Tamil Nadu, India
[2] Department of Zoology, Jamal Mohamed College, (Affiliated to Bharathidasan University) Tiruchirappalli, Tamil Nadu, India

Abstract
Histological research is a useful biomarker for assessing the toxicity of a substance. In this study, the four stages were supplemented with five types of vital pigments (neutral red, rhodamine-B, eosin yellow, acridine orange and light green dyes). Vital dyes were dissolved in distilled water, mulberry leaves were immersed in each dye and stored for a few minutes to evaporate the water that served as the first feed for test larvae. The larvae that received normal leaves (soaked in distilled water) acted as controls. At the end of the test period, the middle intestine was dissected from all groups for histological analysis. The histological evaluation of the H-E colored slide was carried out in response to the observed coloration conditions and to the changes at 40X magnification using the Blisco 111 Pathological Microscope and camera Sony. Based on histological observations of the middle intestine, there was no change between the control group and the various dye treatment groups that did not undergo pathologically significant changes, but which were confirmed by the reconstitution of various mulberry leaves stained with silkworms. The results of this study showed that there is no toxic effect from various supplements of dye mulberry leaves on the silkworm, *Bombyx mori*. This knowledge is crucial in the development of new pigment molecules capable of successfully feeding the larvae of *Bombyx mori* to produce an essentially colored silk in a variety of colors and shades.

Keywords: Histology, silkworm, bombyx mori. neutral red, rhodamine-b, eosin yellow, acridine orange and light green dyes

Introduction
The digestive system is the main system of the animal body, as it is mainly concerned with the supply of necessary nutrients, which are essential for balanced growth, which is the most important part of the body, performing all vital activity and reproduction. The digestive tracts of insects are linear tubes of different diameters in different areas, but some are wound in nature. It eextends from the mouth and ends with the anus It is divided into three regions *Viz*., fore gut, midgut and hindgut which comes from different embryonic origins. The foregut and hindgut is ectodermal in origin while midgut is endodermal in origin. (Borror and De Long, 1969, Belkin, 1976) [5, 3]. Most of the digestive tract is covered with the middle intestine with different types of cells. The cells of the central intestine performance an significant role in the digestion and absorption of nutrients (Cavalcante and Cruz-Landim, 1996, Billinsgley and Lehane, 1996, Cristofoletti *et al*., 2001) [4, 9].
Coloring is the application of aqueous solutions of mainly synthetic organic dyes to fibers, threads and substances. Despite a century of process improvements, the coloring has produced large amounts of toxic wastewater as a by-product. This makes it one of the most environmentally harmful chemical processes and requires efficient and cost-effective wastewater treatment before it can release water naturally (Robinson *et al*., 2001; Moore and Ausley, 2004) [16, 14]. Recently, many synthetic dyes have been used as supplements to produce cocoon colors, such as Rhodamine B, Orange Acridine, Bismarck Brown, Acid Orange 142, etc, and different concentrations of Direct Red (Issa *et al*., 2021) [13], etc. Toxicity assessment of chemicals is a very important procedure that is usually performed before supplementation. Conversely, various methods have been developed and adopted for acute toxicity testing. However, most of these methods have their drawbacks, and now it is important to develop better methods, and if possible, to reduce the number of animals used (Chinedu *et al*., 2013) [8]. In modern times, histology is a tool to access some knowledge about microscopic tissues of organs, the microscopic anatomy essential to understand possible histopathology in diagnosis (Hinton *et al*., 1992) [12]. Since there are relatively few histological descriptions of the insect digestive system in India and these clearly do so, this study should make histological observations of the toxic effects of different dyes (neutral red, rhodamine B, neutral red, eosin yellow, acridin orange and light green dye) in the middle intestine of the experimental fourth and fifth stages of the Mulberry silkworm, *Bombyx mori*.

Materials and Methods
Experimental Procedure
The egg cards of silkworm *B. mori* CSR2 a bivoltin) was obtained from the State Grain Center in Tiruchirappalli, India. Silkworms were grown under standard conditions at 26 ± 2 degrees Celsius. Mulberry leaves harvested in irrigated mulberry gardens were used to feed silkworms. The larvae were grown in plastic trays (30 larvae / tray and only ate mulberry leaves. Fresh mulberry leaves of variety v1 were collected in the early morning and stored in wet hemp bags. It was chopped before feeding. The leaves were fed four times a day (7.00, 11.00 in the morning and 3.00 & 8.00 in the evening hrs).

In the experiments, the fourth stage larvae were supplemented with five types of vital dyes. Vital dyes were dissolved in distilled water and mulberry leaves were soaked in every spot (NR, EY, RHB, AO & LG) and after few minutes after fed to the experimental larvae as the first feed (4th instar day 1). Larvae given normal leaves (dipped in distilled water) acted as controls. All breeding work was carried out according to standard methods. During captivity, the worms were given in six batches of 30 larvae per treatment. At the end of the test period, the middle intestine was dissected from all groups for histological analysis.

Experimental design

The four and five stages of the *B. mori* larvae were used in this study and grouped as follows.

Group I - Larvae provided with fresh mulberry leaves, control.

Group II- Larvae provided with 0.2% Neutral red dye supplemented Mulberry leaves.

Group III- Larvae provided with 0.2% Rhodamine-B dye supplemented Mulberry leaves.

Group IV- Larvae provided with 0.2% Eosin yellow dye supplemented Mulberry leaves.

Group V- Larvae provided with 0.2% Acridine orange dye supplemented Mulberry leaves.

Group VI- Larvae provided with 0.2% Light green dye supplemented Mulberry leaves.

Histological analysis

Histological studies of the intestines of different groups were carried out by the methods of Ochei and Kolhatkar, (2000) [15]. The middle intestine was stored for 24 hours in a 10% neutral buffered formalin solution and 70% washed with ethanol%. The fabric is then placed in a small metal coffin, stirred with a magnetic stirrer, 70% dehydrated with the alcohol series ~ 100% and incorporated into paraffin with a coating machine. The paraffin block is cut with a rotating ultramicrotome, which is distributed on the glass slide. Then it was dried overnight. The slides were dyed under a pathology microscope (Blisco 111), with hematoxylin and eosine dyes (H&E) and examined histological changes after installation.

Results and Discussion

Histology is a microscopic research in which the cells and tissues of animals and plants are dyed and cut and examined under a microscope (electron or optical microscope). There are various methods for examining the tissue properties and microstructure of cells. Histological research is used in forensic examinations, dissections, diagnoses, toxicity and reconnaissance. In addition, histology is widespread in medicine, especially when examining diseased tissues to support treatment (Oh, 2004).

One of the greatest benefits of using histopathological biomarkers for environmental monitoring is the ability to test specific target organs, such as the heart, kidneys, and other organs. The intestine and liver perform important functions such as breathing, excretion, digestion, accumulation and biotransformation of heterogeneous organisms in animals (Gernhofer *et al*. 2001) [11]. In addition, the changes observed in these organs are generally easier to identify than the following functional factors (Fanta *et al*., 2003) [10] and serve as a sign that, that animal health threats warm up (Hinton and Lauren, 1990)). Some heterologous antibiotics initiate the effects of certain enzymes, which also change the metabolism and cause apoptosis, which initially occurs as necrosis with an inflammatory defense reaction (Velkova Jordanska 2002, Roganovic Zafirova *et al*., 1998, 2003) [19, 17]. Histopathological analysis of liver, gills and intestines, vertebrate muscles and kidneys is used to evaluate the belongings of injurious materials and treatments in the ecosystem on their respective animal species (Velkova Jordanaska and Kostoski, 2005) [18].

In the present study, five different types of vital pigments (NR, EY, RHB, AO, LG) were supplemented for four stage larvae. An important dye was dissolved in distilled water. Mulberry leaves were dipped in each spot and left to stand for a few minutes to evaporate the water that served as the first feed for test larvae. Larvae that regularly received leaves (dipped in distilled water) served as controls. At the end of the experimental period, the midgut was dissected from all groups for histological analysis. The histological evaluation of the H-E colored slide was carried out in response to the observed coloration conditions and to the changes at 40X magnification using the Blisco 111 Pathological Microscope and the Sony camera.

Microscopic evaluations

Figure 1 shows the histological observations of Neutral Red (1B), Eosin Yellow (1C), Rhodamine B (1D), Acridine Orange (1E and Light Green (1F) and is compared to control (1A). Histological studies of the middle intestine detected a typical structural composition of the curved structure of the digestive tract. The nucleus of cylindrical epithelial cells was observed in a hardened form. The epithelium consisted of single-row prismatic cells; dark oval microvillus nucleus was observed in contrast (Fig. 1 A). Similar structural arrangements have been observed in other dye treatment groups (Figure B - D). In a microscopic view, the control group shows that the external muscle cells. In the basal part, granular cells are found between the prismatic cells. These cells are regenerative cells. In the epithelial region, stem cell nests were observed, young cells at the base of the epithelium are found individually or several together. They are defined as the region of play (Figure 1 A). Several secretory granules were also found in the cell. External muscle cells, secretory granules, and epithelial cells were also similar to various dye treatment groups. The epithelial cells of the central intestine of insects are generally short-lived and are quickly replaced by regenerating cells between basal-terminal and epithelial cells (Chapman, 1985) [7]

The goblet cells have a pear-like structure based on the basal membrane and have a rounded core that protrudes at the base. The cavity of the goblet cells is lined with apical plasma membranes that form numerous microvilli. Control groups and dye treatment groups had similar caliciform cells located between prismatic epithelial cells, and excretory granules were detected (Figure A - D).

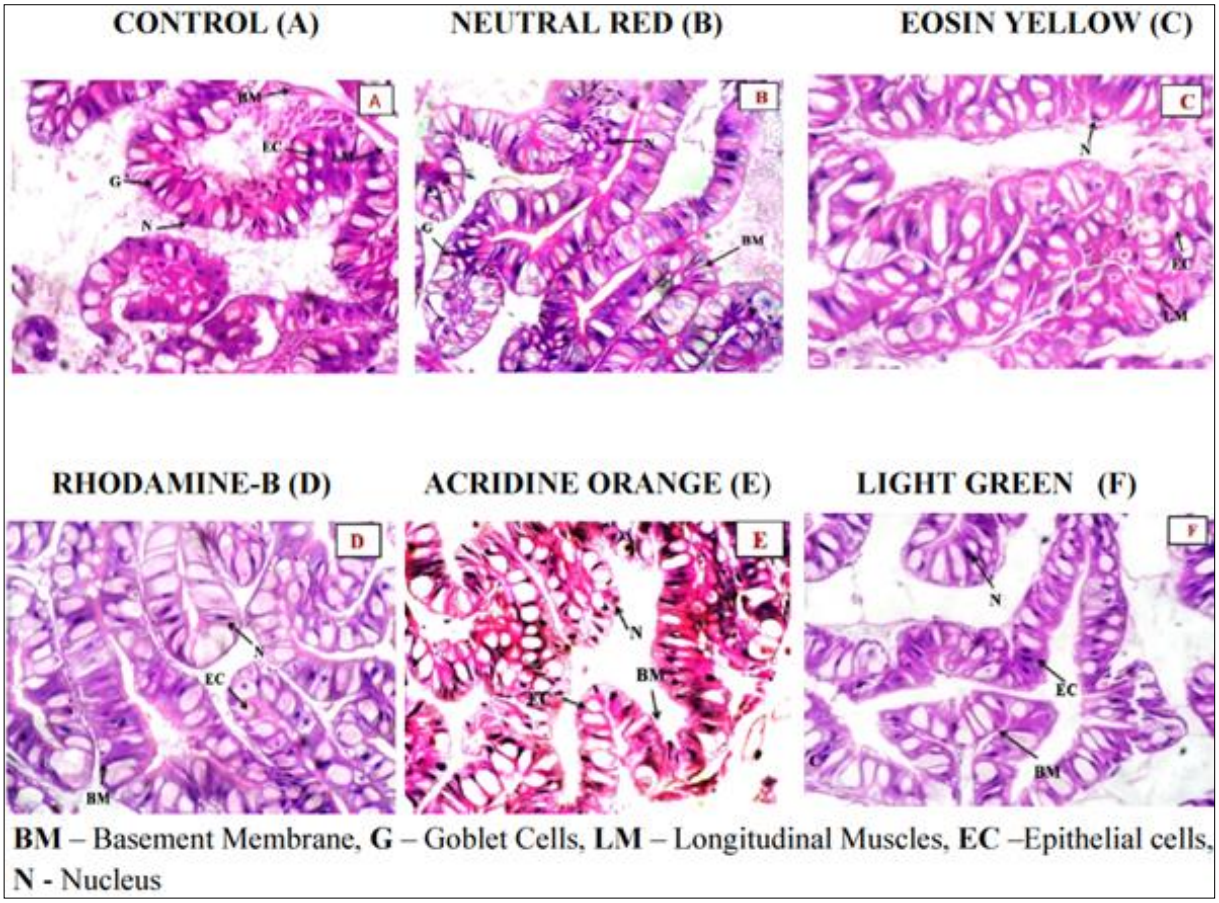

Fig 1: Histological observation of impact of Neutral red (1B), Eosin yellow (1C), Rhodamine-B (1D), Acridine orange (1E), Light green (1F) in control (1A) and experimental midgut of Mulberry silkworm, *Bombyx mori*

Microvilli were also observed. In the basal part of the epithelial cells, the nuclei of the regenerating cells were distinguished. The core was elliptical in shape and strongly colored. Longitudinal muscle cells were also found in the control and dye treatment groups (Figure A - D).

Current research agreement with Anuya Nisa and others. (2014) [1] used an environmentally friendly feeding approach to determine whether the azo dye is absorbed by the silk glands for the production of colored silk cocoons. The results suggest that some dyes produced silk that was essentially colored, while others did not. A careful assessment of the physical properties of these related azo dyes suggests, that the diffusion of dyes from the digestive tract of silkworm larvae requires a balance between the hydrophobic and hydrophilic properties of hemolymphs and later in the silk glands.

Conclusion

Histological research is a useful biomarker for assessing the toxicity of a substance. Based on histological observations of the intestine, there was no change between the control group and the various dye treatment groups that did not undergo pathologically significant changes, but which were confirmed by the reconstitution of various mulberry leaves dyed with silkworms, *Bombyx mori*. The results of this study showed that there is no toxic effect on various supplements of dyes mulberry leaves for the silkworm, *Bombyx mori*. This knowledge is crucial in the development of new pigment molecules capable of successfully feeding the larvae of the *Bombyx mori* to produce an essentially colored silk in a variety of colors and shades.

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