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Hemocyte Changes after the Extirpation of the Hemopoietic Organ-wing Disc Complexes in the Silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)

Qing-xiang Zhou[1,2], Xing-jia Shen[1], Yong-zhu Yi[1], Ai-hua Xia[2] and Zhi-fang Zhang[1,2]*

[1]*The Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang City, Jiangsu Province, 212018, China*

[2]*Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing, 100081, China*

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We successfully extirpated all four hemopoietic organ-wing disc complexes of the fifth instar larvae of *Bombyx mori*, and found that most of the treated silkworms could still develop into the moths. We investigated the changes of the circulating hemocytes and evaluated the effects of extirpation on the hemopoiesis. The results showed that proliferation of circulating hemocytes was sufficient to allow development of the silkworms which complexes were totally extirpated. We also found that hemopoitic organ-wing disc complexes extirpation might cause a certain hemopoietic compensation of the remainder complexes during early spinning. Exogenous hormones such as 20-β-hydroxyecdysone and juvenile hormone analog had a positive effect on hemocytes proliferation.

Key words: *Bombyx mori*; Hemopoietic organ-wing disc complex, Extirpation, Exogenous hormones, Hemopoiesis

Introduction

Hemocytes of silkworm larvae are produced in hemopoietic organs, which are tightly attached to the imaginal wing discs. It is hard to shell off the hemopoietic organ from the wing disc it attaches to, and the two organs are customarily called hemopoietic organ-wing disc complex (we called "complex" for short in the following text) (Akai and Sato, 1971; Nittono *et al.*, 1964). At the beginning of the 5th larval instar, the surface of the hemopoietic organ is covered with a connective tissue sheath. At the end of spinning, the sheath cracks, followed by the discharge of all hemocytes into hemolymph and the disappearance of hemopoietic organs (Sato and Akai, 1977). According to the accepted morphological classification, there are five types of circulating hemocytes in *Bombyx mori*; they are prohemocyte, plasmatocyte, granulocyte, spherulocyte and oenocytoid (Nittono, 1960). It is thought that the former two types are derived from the hemopoietic organs and divide to generate the other three types (Beaulaton, 1979; Nittono *et al.*, 1964; Han *et al.*, 1998; Yamashita and Iwabuchi, 2001).

The importance of hemopoietic organs in the silkworm larval hemopoiesis is shown by their ablation with a hot needle or destruction with heavy ion (Nittono *et al.*, 1964; Tu *et al.*, 1999; Tu *et al.*, 2002). If the organs were destroyed, the hemocyte count in the larval body was reduced. Former studies show that insect hemopoiesis is endocrinally regulated (Hoffmann, 1970; Sorrentino *et al.*, 2002), and that 20-hydroxyecdysone and juvenile hormone III can both induce hemopoiesis *in vitro* (Han *et al.*, 1995; Nakahara *et al.*, 2003). In the present study, we extirpated all of the four hemopoietic organ-wing disc complexes of *Bombyx mori* at the early stage of the fifth larval instar, and investigated the effects on hemopoiesis. Effects of exogenous hormones on the treated silkworm were also examined.

Materials and methods

The animals

The silkworm stock, JY-I, was maintained in our laboratory. During the first four instars, the larvae were reared

*To whom the correspondence addressed
Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, 12 Zhongguancun South Street, Beijing, China. Post Code: 100081, Tel: +86-10-68919854,
Fax: +86-10-68975136, E-mail: zhifangzhang@yahoo.com

on mulberry leaves under standard conditions (Lü, 1990). The newly molted fifth instar larvae were segregated and maintained at 25°C with 70%-80% relative humidity until emergence of the moths.

Extirpation of the hemopoietic organ-wing disc complex
Newly molted fifth instar larvae were sterilized with 70% ethanol and anesthetized in sterile ice water. A small incision was made on the dorsal epidermis near the wing disc location using a scalpel. The complex was taken out from the incision and a blob of low melting point wax was dropped to the cut. The treated larva was maintained at 4°C for about 90 minutes, and then placed to normal condition. Four groups of larvae were prepared: without the right-fore complex (1HWC), without both complexes on the right side (2HWC), with only the left posterior complex (3HWC), and without any complex (4HWC). Intact larvae (CK0) and larvae with a cut in dorsal epidermis away from the complex location (CK) were used as controls. The operations were completed within 8 hours, and all of the experimented silkworms were fed simultaneously.

Hormone treatment
Exogenous hormones were used on one-day old (day 1) larvae which complexes were totally extirpated. Ten microlitre of 0.1 $\mu g \cdot \mu l^{-1}$ 20-β-hydroxyecdysone (MH, prepared by the Sericultural Research Institute, Chinese Academy of Agriculture Sciences) was injected into each larva. Juvenile hormone analog (JHA) ZR512 (kindly provided by Prof. Weizheng Cui from Shandong Agriculture University) dissolved in Tween was diluted to 1 ppm concentration with sterilized water, and then sprayed to the body surface of larvae. Sterile water was used for the control.

Investigation methods
Three to four drops of blood were collected from the first pair of prolegs, by puncturing with a needle, and diluted to a proper concentration with PBS buffer [137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4 and 2 mmol/L KH_2PO_4]. Then the hemocytes were counted with a BOECO blood cell counter (Boeco Germany, Hamburg, Germany), and the hemocyte density was calculated. Silkworm in cocooning was cut out for experimenting. Each treatment was investigated five larvae at least in the experiments. All of the data were analyzed with the SAS Statistical Software.

Results and Discussion

Development of the 4HWC silkworms
The hemocytes of insects are produced in hemopoietic organs and discharged into hemolymph (Nittono *et al.*, 1964; Akai and Sato, 1971). Some scientists have tried to ablate all of the hemopoietic organ-wing disc complexes, but failed due to the death of silkworms supposed by excessive loss of hemolymph (Beaulaton, 1979; Kango-Singh *et al.*, 2001). In our present study, more than sixty percent of the silkworms which four complexes were all extirpated (4HWC) developed normally till eclosion, and the moths remained the ability of mating and ovipositing (Fig. 1). The life duration of those silkworms was the same as that of the controls, though the wounds caused by extirpation were easily bleeding at early pupal stage. The cocoon weight of the 4HWC was about 15% lighter than that of the CK0, while the cocoon shell rate was not significantly lower (Zhou *et al.*, 2004). To our knowledge, we are the first to successfully extirpate all of the four complexes in *Bombyx mori*.

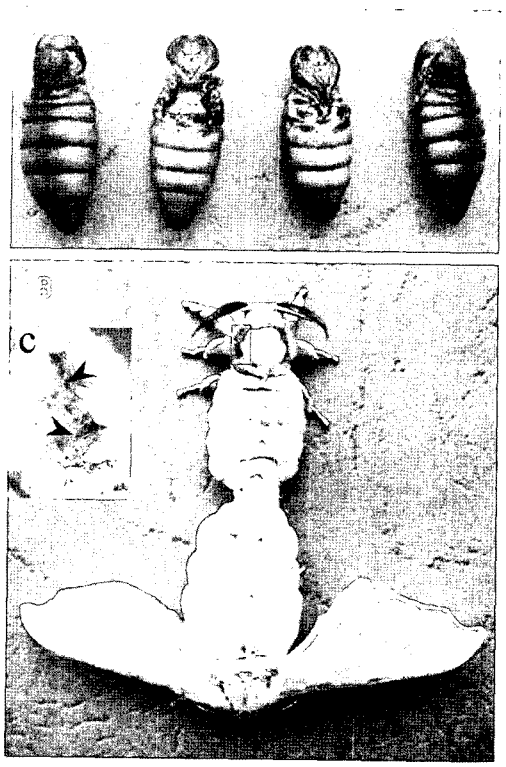

Fig. 1. Pupae and moths of hemopoietic organ-wing disc complexes totally extirpated silkworms. Wings of the pupae are missing (A) and a moth without wings is mating with an intact one (B). (C) shows the enlargement of the boxed region in (B). Arrow heads in (C) indicate the region where the wings should be extruded.

Changes of hemocyte number in the 4HWC silkworms
In order to obviate the interference of bleeding on the results, we set CK besides CK0 in the experiment. The result showed that hemocyte density of CK and CK0 were

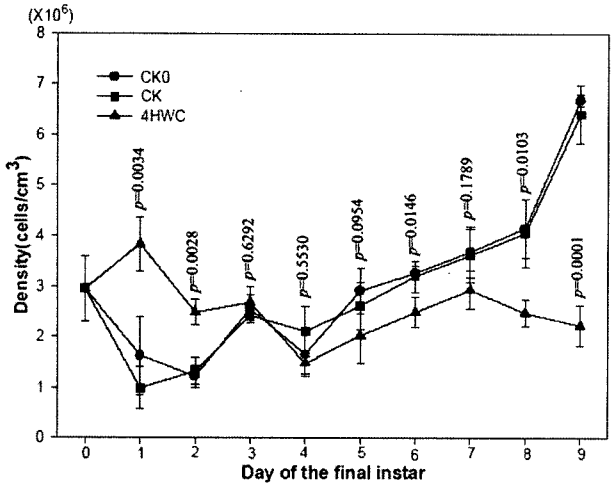

Fig. 2. Hemocyte count in silkworm after the complexes were extirpated. Hemocyte densities of the controls (CK and CK0) are similar ($p>0.05$). While the density of 4HWC is significantly higher at day 1 and day 2, and a little but not significantly lower from day 4 to day 7 (except day 6, $0.01<p<0.05$) than that of the controls ($p>0.05$). But after the onset of spinning (about at day 8, $0.01<p<0.05$), the lowness becomes significant ($p<0.01$ on day 9). CK0, intact larvae; CK, larvae with a cut in dorsal epidermis away from the complex location; 4HWC, larvae without any hemopoietic organ-wing disc complex.

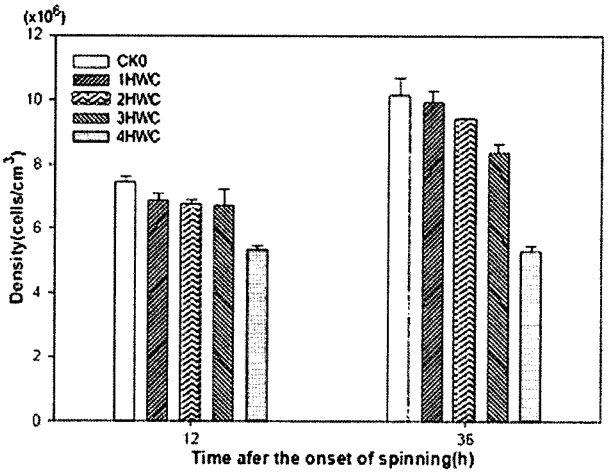

Fig. 3. Extirpation of various numbers of hemopoietic organ wing disc complexes. There are no obviously differences among hemocyte densities of 1HWC, 2HWC and 3HWC ($p>0.05$), and there are no obviously differences between those of CK0 and 1HWC at 12 hours after the onset of spinning. But at 36 hours after the onset of spinning, the more the complexes extirpated, the fewer the hemocytes circulating in hemolympl ($p<0.05$). 1HWC, larvae without the right-fore complexs 2HWC, larvae without both complexes on the right side 3HWC, larvae with only the left posterior complex.

similar ($p>0.05$) (Fig. 2), so we could only take CK0 as the control. Hemocyte density of 4HWC was significantly higher at day 1 and day 2 than that of the control. We thought that this was caused by additional release of unfledged hemocytes during extirpating. But these unfledged cells had a weak vitality and were easy to die, resulting in a rapid decrease of 4HWC's hemocytes in the following two days. A similar result was also reported in studies with heavy ion (Xu *et al.*, 2002). From day 4 to day 7, hemocyte density of 4HWC became a little but not significant lower than that of the control ($p>0.05$ except for Day 6), and after the onset of spinning (at about day 8) it became significantly lower ($p<0.05$). Although hemocyte density of 4HWC was low during most of the time, it played a mild rising trend from day 4 to the onset of spinning. These further indicated that there might be some hemocytes with stem cell properties in hemolymph, and sufficient hemocytes could be produced by mitosis for growth, development and metamorphosis of the complexes totally extirpated silkworms.

Differences among the extirpation of various of the complexes

Normally, during the wandering stage, hemocytes in the hemolymph increase sharply till the top (Sato and Akai, 1977). So we chose wandering stage to investigate the different effect among different kinds of extirpation. Hemocyte numbers of 1HWC, 2HWC and 3HWC were similar at 12 hour after the onset of spinning ($p>0.05$), and there were no obviously differences between those of the controls and 1HWC ($p>0.05$, Fig. 3). The data indicated that there might be some compensation, by promoting the discharge of the hemocytes, for the remained hemopoietic organs. Till 36 hour, the hemopoietic organs cracked and all of the hemocytes discharged into hemolymph. Then, the more the complexes were extirpated, the fewer the hemocytes in body could be found ($p<0.05$).

Hormone effects on hemopoiesis in 4HWC silkworm

The main developmental hormones in insect larvae are the ecdysones (MHs) and juvenile hormones (JHs) (Truman and Riddiford, 2002). Using of exogenous MH or JH can disturb the secretion of MH and JH *in vivo*, and effects the development of the silkworm (Gu and Chow, 2003; Sakurai *et al.*, 1989; Akai *et al.*, 1989). It has been known that exogenous hormones have no significant effects on the hemopoiesis of the intact final instar silkworm larvae *in vivo* (Zhao *et al.*, 2004). Our present data showed that hemocyte density of hormones used 4HWC larvae were fewer than that of none used ones on day 3 ($p<0.05$), while there were no obvious differences between MH and

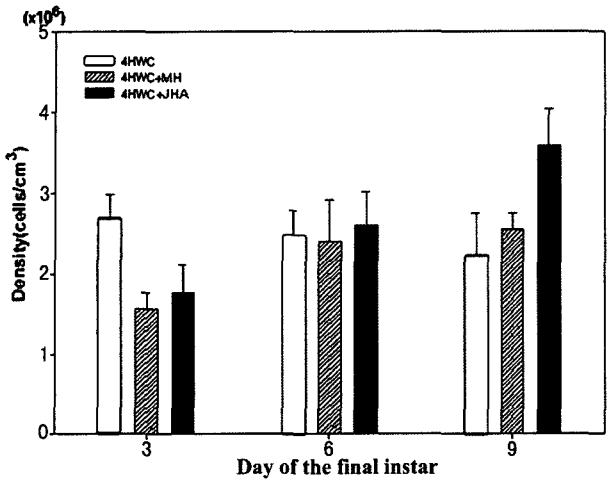

Fig. 4. The effects of exogenous hormones on the hemopoiesis of complexes totally extirpated silkworm. Hemocyte counts of hormone used 4HWC larvae are fewer than that of no used ones ($p<0.05$) on day 3, and of the three treatments are about the same on day 6 ($p>0.05$). But on day 9, it shows that exogenous hormone using has an enhancive function to hemopoiesis (MH, $p<0.05$; JHA, $p<0.01$), and the effect of JHA is significantly higher than that of MH ($p<0.05$). MH, 20-β-hydroxyecdysone; JHA, juvenile hormone analog.

JHA used ones ($p>0.05$) (Fig. 4). It is well known that additional exogenous MH and JHA will postpone the development of the silkworms at the early stage of final instar. This might be the causation of the result on day 3. There even had no differences among the all different treatments in day 6 larvae ($p>0.05$). But on day 9, it displayed an enhancive hemopoietic function of hormone using (MH, $p<0.05$; JHA, $p<0.01$). The enhancive effect of JHA is significantly greater than that of MH ($p<0.05$), i.e., the hemocyte density of the JHA treated 4HWC silkworm was higher than that of the MH treated ones. These showed that the using of JH or MH at the early stage of 5th instar could promote mitosis of hemocytes in 4HWC.

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