Influence of N-methyl-N-nitrosourea Treatment on Embryogenesis of Bombyx mori

Kawaguchi, Yutaka
Laboratory of Silkworm Sciences,Division of Genetics and Plant Breeding,Department of Applied Genetics and Pest Management,Faculty of Agriculture,Kyushu University

Kusakabe, Takahiro
Laboratory of Silkworm Sciences,Division of Genetics and Plant Breeding,Department of Applied Genetics and Pest Management,Faculty of Agriculture,Kyushu University

Koga, Katsumi
Laboratory of Silkworm Sciences,Division of Genetics and Plant Breeding,Department of Applied Genetics and Pest Management,Faculty of Agriculture,Kyushu University

https://doi.org/10.5109/4526

出版情報：九州大学大学院農学研究院紀要. 48 (1/2), pp.59-64, 2003-10-01. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **48** (1·2), 59–64 (2003)

Influence of *N*–methyl–*N*–nitrosourea Treatment on Embryogenesis of *Bombyx mori*

Yutaka KAWAGUCHI†, Takahiro KUSAKABE and Katsumi KOGA

Laboratory of Silkworm Sciences, Division of Genetics and Plant Breeding, Department of Applied Genetics and Pest Management, Faculty of Agriculture, Kyushu University, Fukuoka 812–8581, Japan
(*Received May 12, 2003 and accepted July 15, 2003*)

Embryonic malformation was induced by immersing *Bombyx mori* eggs at 3.5 h after oviposition in a 10 mM *N*–methyl–*N*–nitrosourea (MNU) solution for 15 min. About 80% of thus treated eggs could not hatch. When observed under a light microscope using unsectioned transparent specimens of unhatched eggs, the embryos exhibited abnormalities such as the absence of inner organs, twisting body axis, incomplete body segments and anomalous segmentation. These findings confirmed that the immersing method worked well and MNU strongly provokes various forms of somatic impairment in developing embryos.

INTRODUCTION

In previous articles (Kawaguchi *et al.*, 1985, 1989), we reported that malformation and mutation of *Bombyx mori* could be induced with ease by immersing intact eggs in a solution containing *N*–methyl–*N*–nitrosourea (MNU), one of carcinogenic alkylating agents effective in a wide range of living things (Tazima and Murakami, 1977; Tazima, 1978, 1980). We infer that the eggshell is very porous because of the multiple spiracles or aeropyles penetrating into the egg plasm, thus providing a route through which given chemicals can enter. This situation has made us expect that the silkworm eggs can be exploited as a detection system of environmental chemicals. For this purpose, disturbances of embryogenesis due to chemicals may be a convenient measure, although this point has not been fully investigated in our previous studies, in which major attention was focused on the malformation of larvae surviving beyond hatching (Kawaguchi *et al.*, 1985, 1989). Here, we carried out light microscopic observations of embryos using unsectioned transparent preparations of developing eggs treated with MNU as a model chemical.

MATERIALS AND METHODS

Insects

The silkworm strain used was p22 maintained in the Faculty of Agriculture, Kyushu University (Fujii *et al.*, 1998). The strain p22 is a standard Japanese race, bivoltine tetramolter, producing normal eggs. Full grown larvae of this strain possess normal marking presented by three kinds of spots, i.e., eye spots on the 2nd segment, crescents on the 5th segment and star spots on the 8th segment. Larvae were raised on mulberry leaves

† Corresponding author (E-mail: ykawagu@agr.kyushu-u.ac.jp)

and adults were allowed to mate with each other.

Immersion of eggs in MNU and morphological observation of embryogenesis

Fertilized eggs were collected within 30 min after the onset of oviposition. Eggs at 3.5 h following the end of oviposition were immersed in a 10 mM aqueous solution of MNU for 15 min at 25 °C, washed in distilled water, air dried and kept at 25 °C. Unhatched eggs were collected for observation of embryos, which were considered to be dead (see below). As a control, distilled water was used instead of the MNU solution; the normal eggs were allowed to develop at 25 °C and collected at intervals. All the eggs were treated with hot–HCl at 20 h after oviposition to avoid entering diapause. The collected eggs were fixed with Carnoy's fluid for 3 to 4 days at 0 °C and dechorionated with fine forceps. The whole embryonic specimens with intact yolk spheres were stained with thionine, dehydrated with ethanol, made transparent with benzene, mounted in eukitt and observed under a light microscope. Embryonic lethality was estimated by the following criterion (Kawaguchi *et al.*, 1985); the embryos poorly colored and unhatched were assumed to have died sometime before the body pigmentation stage, while those colored extensively dark but unhatched were taken to have died after the body pigmentation stage, i.e., shortly before hatching.

RESULTS AND DISCUSSION

Rates of abnormality in MNU-treated *B. mori* eggs

The eggs immersed in 10% MNU solution for 15 min at 3.5 h after oviposition exhibited the hatchability of 19.1%, much smaller than the control value of 89.4%. The populations presumed to have died before and after the body pigmentation stage (see MATERIALS AND METHODS for the discrimination criterion) were 22.7 and 58.2%, respectively, of the total MNU-treated eggs, while 0.5 and 10.1%, respectively, in the control. Thus, the total lethality in the MNU-treated eggs amounted to 80.9%, a value very higher than that of the control (10.6%). These results were in agreement with our previous data (Kawaguchi *et al.*, 1985), indicating that the MNU-immersing system elicits highly reproducible responses in the eggs.

Young eggs of *B. mori* are in critical stages of syngamy and initial mitoses (Tazima, 1964). The cell nucleus in the *B. mori* egg shortly after oviposition is in the metaphase II of the maturation division. About 60 min after oviposition, the nucleus enters the second maturation division, and the female pronucleus is made. On the other hand, a sperm staying in the anterior region of the egg matures to the male pronucleus. At 120 min, both pronuclei approach to each other and fuse to complete the fertilization or the syngamy. Thereafter, the synchronous mitotic division starting from the fused nucleus proceeds several times in the yolk mass with one cycle of 60 min. Thus, the largest fraction of the egg nuclei at 3.5 h, when the MNU treatment was performed, must be at the stage between the first and the second mitosis. The above results, together with our previous finding that immersing eggs until 4 to 5 h after oviposition in an MNU solution produces many types of malformed larvae (Kawaguchi *et al.*, 1985, 1989), provided support for the notion that MNU facilely reaches the peripheral ooplasm via the aeropyles. It is inferred that MNU first affects the peripheral ooplasm and then the nuclei.

Morphology of embryos in the control eggs

The developing features of whole embryos in the normal eggs were observed using control specimens (Fig. 1). The germ band is formed on day 1 after oviposition (1). On day 3, the stomodaeum, proctodaeum and appendages appeared accompanying germ–band elongation and body segmentation (2; invagination of trachea initiates at this stage, although invisible in the figure). The embryo exhibits outgrowth, with shortened

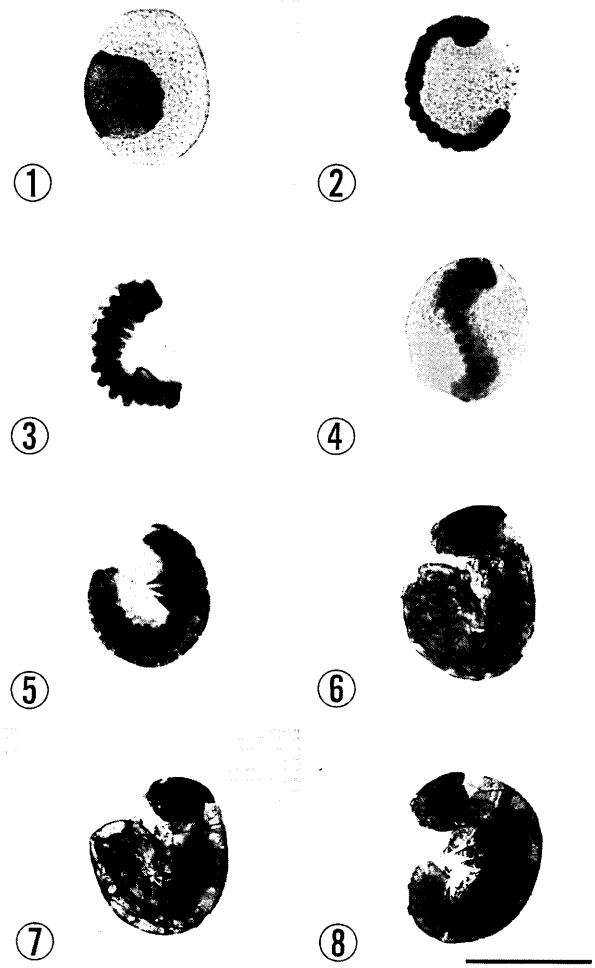

Fig. 1. Microphotographs of embryos of the normal strain p22 of *Bombyx mori* from day 1 to day 10 after oviposition. Panels 1, day 1; 2, day 3; 3, day 4; 4, day 4.5; 5, day 5; 6, day 7; 7, day 9; 8, day 10 after oviposition. Unsectioned transparent preparations were stained with thionine. Scale bar, 1 mm.

labial lobe and differentiated head/thorax regions on day 4 (3). Blastokinesis initiates on days 4–4.5 (4), and is completed on day 5 (5). Pigmentation at the head, seta and epithelium occurs on days 7–9 (6 and 7; the taenidium in spiral band is formed in the tracheal tube). On day 10, the embryo is completed and hatches (8). All these are coincident well with earlier knowledge (e.g., Tazima, 1978; Sakaguchi, 1978).

Morphology of lethal embryos induced by the MNU-treatment

First, the MNU-treated eggs that were presumed to have died before the body pigmentation stage were observed for the whole embryonic features. We found that the serosa remained colorless in many eggs (Fig. 2). It seemed that lethal effects occurred at early developmental stages in these eggs. This category included the specimens with abnormally enlarged cell congeries at one end of an embryo (1), with twisting body axis at both ends of an embryo (2) and with inflated proctodaeum and sparse segmentation but with some appendages being formed (3). Also there were eggs wherein the serosa cells pigmented to some extent (Fig. 3). These embryos seemed to become fatal at later stages than the above-described group. Embryos in these eggs were long and slender (1, 2 and 3), and, at a first glance, looked like day-3 control embryos (germ band elongated and segmented; cf. Panel 2 of Fig. 1). However, all were abnormal, bending at the center and/or end of the body axis and, moreover, being unproperly positioned in the egg chambers.

Then, the embryos in MNU-treated eggs that were considered to have died after the body pigmentation stage were investigated (Fig. 4). In some specimens, the head, thorax and abdomen regions were differentiated like control embryos although neither blastokinesis nor pigmentation at the head, seta and epithelium was complete (1). Sometimes, interior organogenesis was lacking and there were strongly pigmented ectodermal organs alone (2). In the case shown in (3), the embryo developed almost completely but without blastokinesis, thus with appendages being outside.

Embryos died as a consequence of MNU treatment manifested features of a long

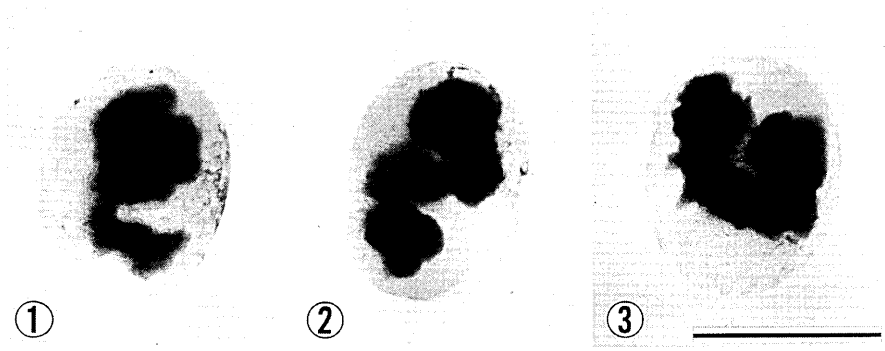

Fig. 2. Microphotographs of MNU-induced abnormal embryos with colorless serosa cells. These were considered to have died before the body pigmentation stage. Panels 1, 2 and 3, different individuals. Unsectioned transparent preparations were stained with thionine. The strain p22 was used. Scale bar, 1 mm.

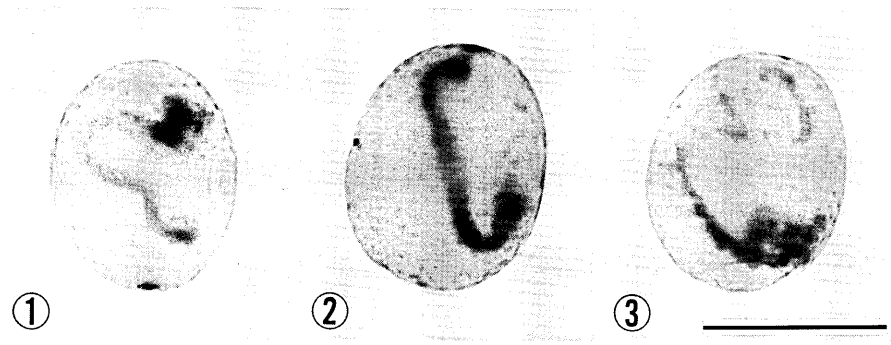

Fig. 3. Microphotographs of MNU–induced abnormal embryos with pigmented serosa cells. These were also considered to have died before the body pigmentation stage. Panels 1, 2 and 3, different individuals. Unsectioned transparent preparations were stained with thionine. The strain p22 was used. Scale bar, 1 mm.

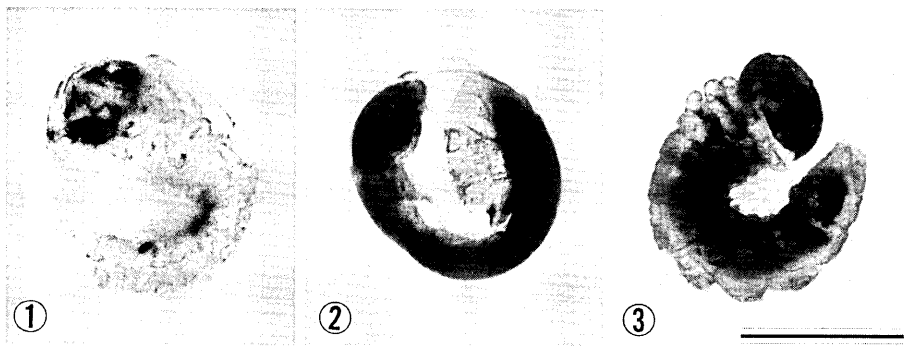

Fig. 4. Microphotographs of MNU–induced abnormal embryos with grown and/or pigmented ectoderm organs. These were considered to have died after the body pigmentation stage. Panels 1, 2 and 3, different individuals. Non–sectioned transparent preparations were stained with thionine. The strain p22 was used. Scale bar, 1 mm.

extent of developmental stages from germ band formation to shortly before hatching. We infer that this chemical makes the cell differentiation in young embryos imperfect, this in turn induces the somatic impairment of organogenesis at the middle to the late stages of embryogenesis and, consequently, prevents hatching of most embryos.

A small fraction of embryos receive a negligible influence by MNU, and can hatch; some of them further grow until the 5th instar, pupal and adult stages, although most are accompanied by various abnormalities such as segment fusion, twist of body axis, deletion and/or surplus of the legs and so on (Kawaguchi *et al.*, 1989). It is concluded that MNU treatment to eggs generates many marked deformities, which affect embryonic and

postembryonic development but may mostly be uninherited to progenies. These findings confirmed the high sensitivities of young B. mori eggs toward active agents, and verified the idea that the egg immersing method we have devised is applicable to the investigation of pathogenic environmental factors that disrupt the development of many organisms.

ACKNOWLEDGEMENTS

This study was supported in part by a Grant-in-Aid for Scientific Research, No.14656024, and for the National Bioresource Project, from the Ministry of Education, Science and Culture of Japan.

REFERENCES

Fujii, H., Y., Banno, H. Doira H. Kihara and Y. Kawaguchi 1998 Genetical stocks and mutations of *Bombyx mori*: Important genetic resources. 2nd ed., Isseido, Fukuoka, pp. 1–54

Kawaguchi, Y., H., Doira, Y., Banno and H., Fujii 1985 Biological effects of N–methyl–N–nitrosourea as revealed by soaking of newly laid eggs of *Bombyx mori*. *J. Seric. Sci. Jpn.*, **54**: 213–221

Kawaguchi, Y., H., Doira, Y., Banno and H., Fujii 1989 Induction of malformation in *Bombyx mori* larvae by immersion of eggs into methyl nitrosourea, *J. Seric. Sci. Jpn.*, **58**: 3387–343

Sakaguchi, B. 1978 Gametogenesis, fertilization and embryogenesis of the silkworm. *In* "The Silkworm: an important laboratory tool". ed. Y. Tazima, Kodansha, Tokyo, pp. 5–29

Tazima, Y. 1964 Biology of the silkworm. *In* "The Genetics of the Silkworm". ed. by Y. Tazima, Logos Press, London, pp. 1–17

Tazima, Y. and A. Murakami 1977 Appraisal of silkworm oocyte system for mutagenecity testing of environmental chemicals. *Ann. Rept. Natl. Inst. Genet. Japan.* **27**: 259–256

Tazima, Y. 1978 Mutagenecity testing of environmental chemicals. *In* "The Silkworm: an important laboratory tool". ed. Y. Tazima, Kodansha. Tokyo, pp. 247–278

Tazima, Y. 1980 Chemical mutagenesis in the silkworm. *In* "Chemical Mutagenesis". ed. E. J. de Serres, A. Hollaender. Plenum Publ. Corp., New York, pp. 203–238