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Review

Overview of silkworm pathology in China

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In this study, we elaborated the history and progress of studies on silkworm diseases in China through summarizing and reviewing the achievements on silkworm pathology, pathogenic molecular biology, epidemiology, pathogen detection and diagnostic techniques, damage from non-infectious silkworm diseases and control of non-infectious silkworm diseases. These summaries and reviews would provide good references for further studies in silkworm diseases.

Key words: Silkworm, pathology, disease control, China.

INTRODUCTION

Sericulture, originated in China 5,000 years ago, is one of the traditional industries in China. There is a profound cultural foundation and broad market prospect. According to historical records in the word, silk is not only as a kind of commodity, but also as a kind of art form spreading from China to other countries. It has played very significant roles in promoting economic, trade and cultural and art exchanges between China and many other countries. At present, sericulture is still one of the important economic pillars in promoting the adjustment of rural industry structure, eco-environmental construction and increasing the income of peasants and national export earnings in China. More importantly, sericulture has become a major practice in the western area of China for the nation's industrial structure adjustment. It has been one of the primary industries to alleviate poverty of western farmers.

Since 1970s, cocoon production in China has developed rapidly. In the late 1970s, China's output of silkworm cocoon and raw silk completely surpassed Japan's, being the world's largest silk producer. Based on "China Silk Year-book 2007", the world's total silkworm cocoon production was 809,991 tons. Out of them, 621, 052 tons was produced in China, accounting for 76.7% of all production in the world. After then, cocoon production in China in 2009 reached 575,300 tons. In 2006, the exports of raw silk in China was $ 8 644 000 000. China's raw silk exports played a leading role in the internationa l market. It has been dominating the international market market prices gradually and forming the international center of cocoon, raw silk and related trade information (China, 2007; Silk magazine, 2008). The silk industry became one of the competitive industries in China at present and also a predominant producer in the international silk market after China's accession to the WTO.

Although, China has been the world's largest silk producer since the late 1970s, silkworm disease has become one of the greatest threats facing by the sericulture. The damage due to silkworm disease created loss about 10% every year (Huang, 2003). On the other hand, the environmental chemical factors on the poisoning continue to occur. This phenomenon had a significant impact on the stability of the sericulture, also on our harmonious society, so that studies on silkworm pathology and applied researches are very important in the sericulture. As the applied research, silkworm pathology is the field of pathogen and control of silkworm diseases based on laws of basic sciences, played a positive role in China sericulture's development of technology and production.

The studies on silkworm pathology in China had a long history. China has made a great progress in silkworm pathology. Since the founding of People's Republic of China, especially after the implementation of "Reform and Opening-door Policy", some aspects of this research field have reached the world's top level. The researches include silkworm pathogen, pathogenic molecular biology, epidemiology of silkworm diseases (pathogenesis, pattern of contagion), silkworm disease inspection and diagnosis, silkworm disease prevention and control technology (control procedures and pharmaceutical development), diagnosis and prevention of non-infectious silkworm diseases, inheritance of disease resistance and

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breeding of disease resistant silkworm varieties, etc.

SILKWORM PATHOLOGY: CONCEPT, CONTENT AND HISTORY

The concept of silkworm pathology and types of silkworm diseases

Silkworm pathology is a subject of applied science which studies the silkworm (*Bombyx mori*) etiology, pathogen characteristics and pathogenesis, transmission and diagnosis, prevention and control principles and methods. Silkworm disease is a phenomenon of physiological disorders caused by invasion of pathogenic microorganisms and parasites, or influence of physical and chemical factors and other adverse environmental factors, showing a variety of abnormal conditions or death of the silkworm.

There are many kinds of silkworm diseases, each of which has different symptoms. According to causes of the diseases, they are divided into infectious silkworm diseases and non-infectious silkworm diseases. The infectious silkworm diseases are caused by pathogenic microorganisms or protozoon infection. It can be divided into 4 classes, namely virus disease, bacterial disease, fungus disease and protozoa disease. So far, four virus diseases have been found. They are nucleopolyhedrosis, cytoplasmic polyhedrosis, viral flacherie and densonucleosis. Bacterial diseases include septicemia, sotto disease and bacterial intestinal disease. Fungal diseases were found to have white muscardine, green muscardine, yellow muscardine, grey muscardine, black muscardine and aspergillosis. Protozoa diseases include pebrine and amoeba disease. These diseases can infect healthy silkworm larvae via diseased larvae, pupae or their excreta. Non-infectious silkworm diseases are mainly caused by arthropods, toxic substances and physiologicaldisorders, etc.

Research history of silkworm pathology

China has a long history in the prevention of silkworm diseases. Back in the early Spring dynasty, a politician named Guan Zhong (-645 BC) in his writings ("Guanzi Shanquanshu") had recorded the silkworm diseases' damage to the production. Later in the year of Jin dynasty as the "Chen Nongshu" and "Nongsang Jiyao " in the Yuan Dynasty and "Wuben Xinshu" and "Huchanshu" in the Qing Dynasty have recorded silkworm diseases respectively about species, pathogen and symptoms, enhancement of the breeding administration and noticed that clean environment was very important for silkworm rearing. During the Song dynasty, Chen Fu in his book "Agricultural Book" recorded that the occurrence of the stiff disease and the relationship with the temperature. In Ming dynasty, people had a preliminary understanding to the grasserie diseases. In the book "Tian Gong Kai Wu", the symptoms of nuclear polyhedrosis had been recorded. However, due to the slow development of China's basic scientific research, at that time people had no idea to ascertain the real pathogen of silkworm disease. China also started the research on using drugs to prevent and control silkworm diseases very early. In the Qing dynasty, the book "Sang Chan Shi Lu" had recorded disease control methods for 24 different kinds of silkworm diseases and suggested to use fresh lime for disinfection of silkworm rearing seats (silkworm epidemiology, 1986 ver). Modern research and prevention of silkworm diseases began in 1897 in Hangzhou, Zhejiang Province, after the found of the Silkworm Science School. It began to use the microscope for pebrine inspection. From 1920s, Nanjing Central University, Zhejiang University in Hangzhou, Lingnan University in Guangzhou, Zhongshan University, Jiangsu and Zhejiang provincial sericultural experimental farms had been successively conducting studies on silkworm disease prevention. It was the start point of a history of Chinese silkworm disease prevention professional research. After 1949, the East China Sericultural Research Institute (now called Sericultural Research Institute of Chinese Academy of Agricultural Sciences) was established and the Department of Silkworm Pathology was set up in 1951. This department had made a great contribution to the research on virus diseases, virology theoretical and applied studies and prevention of fungal diseases. Researches on cytoplasmic polyhedrosis disease were initiated in 1956. For the first time, the pathogen of infectious flacherie was proved to be non-inclusion body virus by experimental means in 1959. In early 1960s, China began to study the relationship between virus disease and factors of pathogens, environmental condition and the physiological condition. The researchers also systematically studied the mechanism of silkworm diseases "triggered" by environmental factors. They carried out the genetic researches of silkworm's resistance against viruses in 1964. At the same time, more than twenty provinces and autonomous regions had set up the sericultural research institutes and had a pathology laboratory for silkworm pathological study. 9 colleges and universities had also established professional silkworm disease research groups, forming the national silkworm disease research network and starting the systematic research of basic theory and application of silkworm diseases (Acta, 1990).

SILKWORM PATHOLOGY

Silkworm disease etiology

Pathogens of virus diseases

In 1956, China carried out the study on cytoplasmic polyhedrosis disease (CPV). In 1959, Chinese researchers discovered that silkworm infectious flacherie was caused by highly infectious viral pathogen without inclusion bodies for the first time in the world. During late 1970s, researchers from the Sericultural Research Institute of Chinese Academy of Agricultural Sciences confirmed that this pathogen belonged to parvoviridae, named densovirus, which was different with

densovirus Ina isolate found in Japan (Qian et al., 1985). Then they had done systematic studies on symptoms, infectious and biochemical properties of silkworm densonucleosis. They had characterized the densovirus using 7 different samples collected from different areas and made a conclusion that the nucleic acid of densovirus disease virus was single-strand DNA, further confirming the pathogen of silkworm densonucleosis disease as densovirus (Chang et al., 1982; Dairen et al., 1982; Huxue et al., 1983). They studied the difference between densovirus and bacteria and distinguished viral softening disease (infectious flacherie) and bacterial softening disease (Lirong et al., 1981). In Japan, researchers also found different strains of densovirus, defined as Yamanashi, Saku and Ina isolate. Of which Yamanashi and Saku isolate were found to have serological relationships with China isolate. Researchers found that densovirus was double strand RNA virus, which could be divided into 10 fragments. The result of comparative analysis of fragments S6 and S7 from double-stranded RNA of Bm2CPV21 showed that S6 was 1,796 bp and S7 was 1,501 bp long, encoding 561 amino acids and 448 amino acids, respectively (Hagiwara and Matsumoto, 2000). Fragment S8 from BmCPVI strain and H strains respectively were 1,328 bp in same size, encoding 390 amino acids with a molecular weight of 44 kDa. It was the nonstructural protein of BmCPV. The identity of amino acid sequences reached up to 98% (Hagiwara et al., 1998). Fragment S9 from H strain and I strain contained 1,186 bp, encoding 320 amino acids. There were only 37 bp and 6 amino acids different (Hagiwara et al., 1998). Comparing hexagonal polyhedron and square polyhedron, there was only one amino acid different. They analyzed the genomic DNA and molecular diversity of BmNPV, Zhejiang strain and found that there was only one amino acid different between BmNPV Suzhou polyhedron mutant and BmNPV normal hexagonal polyhedron strain (Gong and Keng, 1993). Xuxushi et al., (1994) discovered that the deformed polyhedra mainly appeared in square polyhedral BmNPV and there were some chimeric type and defect type only in deformed polyhedra. The rate of former was high than that of the latter (Xuxu et al., 1994). Mechanism of replication initiation of BmNPV DNA was studied (Zhi-Fang et al., 1995). P10 gene of BmNPV was cloned and sequenced (Yao-Zhou et al., 1992) for constructing the general transfer vector (Zhang and Xiang-Fu, 1994). DNA polymerase gene (Zhi-Fang et al., 1995) and helicase gene (Zhi-Fang et al., 1994) of BmNPV were cloned and sequenced in full length or partial. It was preliminary proposed that the conservative 13 bp stem ring structure of hr3 was the starting point of the core or recognition element (Zhu, 2002). Successive cloning and structure function analysis was conducted to characterize BmNPV genes including egt gene (Ji-ping, 2000) lef - 1 gene (Ji-ping et al., 1997), DA26 gene (Ji-ping et al., 1997), ORF75 gene (Jiang, 2006), SOD gene (Wen-Bing et al., 1999) and PTP gene (Guo, 2001) etc. BmNPV was used as carrier to express a variety of foreign genes in silkworm larvae efficiently (Cuihong and heng, 1999). Researchers also found that mutants of BmCPV and BmNPV, in which mutant M of BmCPV formed polyhedron in intestinal cylindrical cellular nuclei and mutant A of BmNPV formed polyhedron in cellular nuclei. The H.strain which formed polyhedron in cytoplasm had two amino acids different in the primary structure (Kansai Branch of Japanese Silkworm, 1990). Xinyao Xu et al., (1996) found that square polyhedron mutant (GCPVt) and hexagonal wild strain (GCPVh) of BmCPV had strong pathogenicity. There were differences in polyhedral ultrastructure, viral particles of embedding and electronic density of polyhedral protein (Xingyao, 1996). The latter strain, which was a square polyhedral strain, is one of BmNPV mutants (BmNPVt). Comparing with the hexagonal strain, BmNPVh, there was no significant difference in symptoms of infected silkworm and in the virus latent period, cell pathological changes, the target tissue, virus proliferation and virulence. However, they form two different capsules (Gong and Youliang, 1991). Japanese scholars successfully separated temperature-sensitive mutated strain, named as NPVts, in the inducer existence condition (Hashimoto, 1995) and conducted comparative analyses between China DNV isolate and Japan Yamanashi isolate in protein structure and organization, molecular weight, virus characters, sequence of nucleotides, sequence of amino acids (Guang-zhi et al., 2004), DNV organization parts and food under parasitic infection parts and process. They concluded that the virus parasitic site is in the anterior and posterior intestine cylindrical cell nuclei, not parasitic in other tissue cells (Guoxi et al., 1985). DNV infects the intestinal infection first and then gradually hinds infection proliferation in central, anterior intestine to spread the infection. After infection, silkworm of DNA, RNA bowel increasing concentration decreased in the epidermis and pure amine bowel and corruption amine than health (Naka et al., 1993), less silkworm. The nucleopolyhedrosis virus proliferation of inhibiting was also studied. RNAi mediated inhibition can inhibit the corresponding RNA components by genetically modified to host and make host produce certain resistance. Through the duplicate gene ie-1 and lef-1 with appropriate design of RNA interference section and constructing the corresponding transgenic carrier into silkworm BmN cells, the transient expression results showed that carrying with RNAi qualities of the cells are genetically modified. The carrier BmNPV has certain inhibition to virus. Among them: IE dsRNA had inhibitory effect at virus titer of 10^{-4} and had better effect at virus titer of 10^{-5}, while dsRNA LEF had inhibitory effect at virus titer of 10^{-5} and had better effect at virus titer of 10^{-6}. By genetically modified and G418 screening, genetically modified IE dsRNA cells had varied inhibition effect at virus titer of 10^{-4} and better effect at virus titer of 10^{-5}. Resistance difference of transgenic LEF dsRNA cells was observed at virus titer of 10^{-5} (Xueren et al., 2008). According to the replication essential genes ie-1 of BmNPV, a dsRNA was designed and integrated into a transgenic carrier piggyantiIE-Neo containing the ie-1 dsRNA express box. The results showed that stably transformed Bm cells expressing the short ie-1 dsRNA could inhibit the proliferation of BmNPV. Reverse PCR analysis showed that in the transformed cells, the exogenous DNA fragments had been inserted in the cellular genome through random integration or

according to piggyBac specific target site typical of TTAA site (Zhang et al., 2008).

Pathogens of pebrine

In China, pebrine microsporidia have been isolated from silkworm larvae and moths and from field insects including *Phyllobrotica armata* (Liao et al., 1992), *Cerace stipatana* (Sericulture Research Institute of Sichuan Province, 1987), *Hemerophila atrilineata* (Meiling and Jin, 1989), *Calospilos suspecta* (Shen et al., 1996) and *Pieris rapae* (Zheng et al., 1997). The isolated microsporidia belong to genera Nosema, Vairimorpha, Endoreticulatus and Thelohania, respectively. The differences of these microsporidia in shape and size, parasite location and the pathogenicity to silkworm were investigated (Zheng et al., 1997; Fangding et al., 1991; Wan et al., 1995; Zheng et al., 1999). Yongzhen et al., (1999) conducted detailed studies on ultra-structures of several species of microsporidia, showing the differences in internal structure of the spore wall layers, polar tube structure, the similarity of the fixed board, most membrane structure, cells of the posterior pole, the internal structure of polar tube turns and tilt angle, the number of ribosomes and arrangement between different microsporidia . Nowadays, studies on microsporidia have entered the mole- cular level. Microsporidian DNA extraction methods (Cao et al., 1995; Chen et al., 1996; Chen, 1997 Zhang et al., 1995) and RNA preparation methods (Wang et al., 2000), had been established. Partial sequences of microsporidia have been cloned and sequenced (Zhang et al., 1995). Genes from *Nosema* RAD37 (RAD51 homolog) were cloned, expressed and *in vitro* translated (Gao et al., 2001). *Nosema* DNAs were detected by PCR (Chen, 1997) and RAPD markers (Jiaping and Chen 2003; Hong and Duan, 2001). Cloned *Nosema* cabbage butterfly small subunit ribosomal RNA (SSUrRNA) core sequence of the gene was analyzed (Wang et al., 2001). The RNA (SSUrRNA) coding genes and secondary structure was investigated (Wang et al., 2002). Pathogenicity of two new *Nosema* (SCMs and SCM) strains of silkworm were analyzed (Wan, 1998). *Nosema* surface protein extraction method was developed (Cui et al., 1999). Evolution of *Endoreticulatus bombycis* was analyzed based on the rRNA sequence (Pan et al., 2003). Total protein and surface protein of microspores were analyzed (Guo et al., 1995). *Nosema* chemical properties of proteins (Yongzhen et al., 1999) and surface antigen on the pathogenicity of *Bombyx mori* (Cui et al., 1999) and changes in surface protein (Chen et al., 2007) were investigated. *B. mori* bacterial artificial chromosome library construction research was conducted. Selection of restriction enzymes, spores of the processing and preparation of DNA inserts, ligation, transformation and other techniques were explored and optimized (Yang et al., 2009).

Bacterial pathogens

In 1970, the Sericultural Research Institute, Chinese Academy of Agricultural Sciences, which firstly identified the case of "late molter disease" in production was a bacterial septic-mia. Devdas et al., (1994) analyzed fat and protein changes in hemolymph and intestinal of the 5th instar silkworm larvae after being inoculated with prodigiosin (*Serratia marcescens*). It was found that content of carbohydrates decreased in intestine and increased in hemo- lymph (Sam et al., 1994). FuKuda proved that enterobacteriaceae (*Enterobucter* sp.) isolated from mini-spot tiger moth of mulberry had infectivity to silkworm. The bacteria divided in the fat body, trachea and other tissues (FuKuda and Lwashita, 1988). Hideshi (1993) studied δ2 endotoxin CryIA (a) of *Bacillus thuringiensis*. Its toxicity to silkworm was found to be 17 times higher over CryIA (b). And the trypsin activated CryIA (a) and CryIA (b) had specific affinity to silkworm midgut velvet-like capsule (BBMV) and saturated binding property. Both of them competed for the same binding site. Toshihiko et al. (1994) discovered CryIA (a) gene in subspecies sotto of *Bacillus thuringiensis* and CryIB gene of which the encoded protein also had insecticidal activity.

Fungus pathogens

Yanagi (1987) analyzed process of the pathogen *Beauveria bassiana* infecting 2nd star silkworm larvae and found that the hypha infected the larva midgut from intercellular space and did not directly from midgut tissue (Yanagi and Lwashita, 1987). Jijiong Cai (1989) analyzed the fungus growth and proliferation in silkworm body after 12 to 24 h postinfection by inoculation of the *B. bassiana* conidium onto the body surface (Cai 1989). Zhuli Zhi (1988) tested pathogenicity for the two fungi, *Paecilomyces fumosaroseus* and *Cephalo-sporium lecanii* to silkworm (Zhuet al., 1988). Fangrong Zhu (1997) indicated that *Paecilomyces farinosus* was one of the pathogens in gray stiff disease by injected the fungus into silkworm (Zhu et al., 1997). Qiji Zhou (1993) studied pathogenicity of the *Aspergillus versicolor Tiraboschi* to silkworm and found that this fungus produced a toxic metabolin, multiple-colorred asperqillin (Zhou, 1993). Wang (1987) found that *Fusarium* is one of the pathogens of silkworm blacked tail disease (Wang et al., 1987). Quan (2001) indicated that the blacked tail disease occurred because of the *Fusarium* infection; however, the infection rate was very low. In natural condition, the blacked tail disease might occur because of co-infection of *Fusarium* and *Aspergillus* (QIan et al., 2001).

The epidemiological study of silkworm

Back in the early 1960s, China has already studied the relation of the pathogenic virus, environmental conditions and physiological state of the silkworm and the pathogenesis of the environment, studied the environment "induced" diseases of silkworm systematically, proposed the new concept of “induced” disease caused by small quantity of virus under adverse environmental conditions (Institute and silkworm, 1984). Sericultural Research Institute, Chinese Academy of

Agricultural Sciences, conducted a series of studies on pathogens in flacherie diseases, including the latent period of infection and relationship between environmental factors and virus occurrence, suggesting that stimulating factors could cause weakness and silkworm physiological disorder which further led to infection from silkworm viruses. The incidence of viral diseases and the softening of intestinal bacteria which is closely related to the presence of bacteria would accelerate onset of the viral disease (Huxue et al., 1983; Wang et al., 1964; Cao et et., 1965a, b, 1966). In 1978, the Silkworm Research Institute of Guangdong conducted studies on the difference of resistance of different silkworm species to NPV and the inheritance pattern (Silkworm disease group of Guangdong silkworm Institute, 1979; Meng, 1982). In 1979, Huang studied the resistance differences of different species to CPV (Huang, 1979). In 1982, the Sericultural Research Institute, Chinese Academy of Agricultural Sciences, studied the resistance of different varieties to DNV, discovering that the resistance was controlled by a recessive gene (Hu Xue et al., 1984; Hu Xue and Qian, 1981; Qin and Wenzhong, 1988). It was found that under different physiological and ecological conditions (different temperatures, different nutritional conditions, chemical stimulation of different reasons); the difference of viral resistance was significantly related to age of the larvae. As the larval development advanced, the resistance was increased remarkably (Youliang 1983, 1986a, b, c, 1989, 1987). Studies also found that in the occurrence of muscardine diseases, the temperature of infection mainly influenced time from infection to onset of the disease and humidity mainly influenced incidence rate of the disease. Different varieties had different resistance to muscardine diseases. Silkworm varieties of Chinese strain showed symptoms earlier than those of the Japanese strain (Guo and Junliang, 2000). Researchers showed that existence and reproduction of viruses, fungi, microsporidia and other environments had certain patterns in silkworm raising environment. Existence and distribution of fungal pathogens in the air had close relationship with the occurrence of silkworm fungal diseases (Huang, 1988). Yellow muscardine (*Isaria farinosa*) had two occurrence peaks in June and September, respectively. *B. bassiana* was similar to *I. farinosa*. *Aspergillus flavus* was the most widely distributed, which was more likely to occur in July. In this period, silkworm also had high incidence of fungal disease (Shi and Tao, 1994). Studies on the infection process, occurrence period, onset of midgut grasserie showed that occurred in the summer and autumn silkworm intestinal abscess of the pathogen mainly from silkworms of spring rearing season (Huang and Youhua 1983, 1986, 1987, 1988a, b). Virus, pebrine disease, fungal diseases and other pests existed cross-infection between silkworm and mulberry pests. Besides silkworm pebrine pathogen *Nosema*, microsporidian from other field insects, especially the insect in mulberry field also had the opportunity to infect silkworm. Therefore, strengthening management, perishing mulberry pests and cleaning up the environment could reduce or control occurrence of *Nosema* infection (Chen, 1989).

The research of the detection and diagnosis of the silkworm disease

Since 1949, various serological techniques have been gradually and widely used in the study of silkworm diseases. Jianguo Chen et al. (1989) prepared NPV, CPV and DNV rabbit anti-serum and anti-DNV monoclonal antibodies (Coyama, 1989). By double diffusion and immunoelectrophoresis methods, it was showed that densovirus of China Zhenjiang isolate had serological affinity with Japan Saku, Yamanashi and Ina isolate (Lirong, 1981). Chiaki et al. (1987) conducted studies on virus identification, detection, location and early diagnosis by means of agar double diffusion, indirect enzyme antibody (IIP method) diagnostics (Coyama, 1989), latex agglutination (Hiroshi, 1989), conjugated monoclonal antibodies to latex protein A (PALMAL) (GuoXi et al., 1989) counter immuno electrophoresis (Xuefang et al., 1979; Qian et al., 1981 Jin, 1987), immunofluorescence, ABC2ELISA method (Shi et al., 1989), reverse indirect hemagglutination reaction (Huang et al., 1979) and immunological binding assay (Guo et al., 1988). At the same time, they extended the agar double diffusion, counter immuno electrophoresis, latex agglutination serological and some rural areas of early diagnosis in sericulture production. DNV has also been prepared into a diagnostic kit for the silkworm and counter immuno electrophoresis with enzyme (ELCIEP) (Wang et al., 1983). Jianguo et al. (1992) combined the monoclonal antibodies and other immunological technologies with two-way diagnosis of silkworm densovirus. This monoclonal antibody and immunoenzyme technology or combination of fluorescent antibody technique could detect virus in the midgut of silkworm in 16 TO 18 h after infection (Zhao et al., 1990). Zhao et al. (1990) prepared antigen anti-serum and monoclonal antibodies to resist *Nosema bombycis* prepared spore surface (Qian et al., 1986) (year) detected *N. bombycis* by indirect immunofluorescence method (Qian et al., 1986), latex agglutination, enzyme antibody method and carbon agglutination (Liu et al., 1995). Liu et al made new constructs with anti-*Nosema bombycis* monoclonal antibody immunogold silver staining (IGSS), which was used to detect *N. bombycis* (Liu et al., 1995; Xingyao et al., 1998). Serological techniques such as when the root is also used to study silkworm pathogenic fungi. Conidia and blastospores of silkworm white and yellow muscardines had good antigenicity, in which blastospores were better than the conidia. White muscardine blastospores and yellow muscardine blastospores had immune cross-reaction (Liangen and Tao, 1994).

With modern molecular biology techniques and methods of application of the silkworm pathology, some of the silkworm pathogen detection has entered into molecular level. PCR (polymerase chain reaction) has been used to diagnose *B. mori* nuclear polyhedrosis virus (Naxin et al., 1994). The hemolymph sample of infectious Bm2NPV displayed a clear 240 bp fragment from PCR amplification. PCR could also be used to detect BmNPV and DNV from the feces of silkworm (Naxin et al., 1994; Abe et al., 1993). PCR technology for *Nosema* species-specific diagnosis could distinguish the MG1

and MG2 strains of *N. bombycis* from other microsporidia. It could also be used to detect *Vairimorpha necatrix* and *Pleistophora anguillarum* in tussah silkworm, with sensitivity to 1 ng level (Abe et al., 1993; Cai et al., 1997). RAPD (randomly amplified polymorphic DNA) could be used to discriminate different sources of *Nosema* from showing the genomic DNA polymorphism (Wang and Huang, 1999).

Non-infectious disease of silkworm and control of hazards

The research of hazards of fluoride on the silkworm

In recent years, with the development of rural industry, industrial emissions and car exhaust, environmental pollution has become increasingly serious. The emission of fluoride has caused harm in many silkworm raising areas. Mulberry is a plant very easy to absorb fluoride gas and to accumulate fluoride in it, in addition to the endogenous fluoride it has. Experiments proved that for the 3rd instar silkworm larvae, the mulberry silkworm fluoride content of 30 ppm was the security value. When the 1st to 2nd instar silkworm larvae were treated with fluoride in excess of 50 ppm, they would be poisoned Tang et al. (1984) and the higher the fluorine content, the heavier the degree of intoxication and more serious the losses. Yet, different silkworm varieties had different tolerance to fluoride. Generally, silkworm varieties for summer and autumn rearing had higher resistance than those for spring rearing (Chen, 1982; Wang et al., 1991). Silkworm variety Feng 14A had very strong resistance to fluoride (Sericultural Research Institute of Chinese Academy of Agricultural Sciences summer breeding group, 1988). Feeding silkworm larvae with fluoride contaminated mulberry leaves significantly decreased silkworm's resistance to fluoride (Youliang, 1984). The domestic and foreign fluoride study of the effects of silkworm has reached a certain level. After eating mulberry leaves contaminated by fluoride, content of hemolymph calcium and magnesium decreased in the larvae, lipase activity increased and the activity of glutamyl-alanyl-transaminase and protease was also affected (Lin et al., 1994). In the intestine, activity of a-type and b-type glycogen phosphorylase was significantly decreased. Especially, b-type activity decreased greatly, which inhibited sugar catabolism in larval midgut and thus, reduced the cell's energy supply (Shun et al., 1994). As in the intestinal epithelial cell damage, membrane Ca^{2+}-ATPase activity was affected. Ca^{2+} concentration in hemolymph was reduced and H^{+} and Na^{+} concentration was increased (Zang and Shunlin, 1995). Silkworm hemolymph superoxide dismutase (SOD) activity was also affected by fluoride (Xiaofeng et al., 1997). After silkworm ate mulberry leaves treated with fluoride, the body wall and the intestine were both damaged. The epithelial cells of body wall showed vacuoles, dermal necrosis after deposition of hemocytes in the epidermis, with dark brown granular material in the aggregation the formation of dark brown spots (Zhou et al., 1997). Significant lesions in the midgut cell layer relaxation, intracellular vacuoles, mitochondria cavitation, expansion of the endoplasmic reticulum, nuclear membrane degradation, nuclear transfer cemented lumps, lesions in the midgut of silkworm body seriously affect digestion and absorption, so that silkworm malnutrition, body lean, physical weakness, severe dissolution of silkworm with cell death (Zhong, 1994). Fluoride can cause cells in the midgut of the silkworm rough small cell body and mitochondrial degeneration and finally collapse completely in the intestinal cells, intestinal alkaline phosphatase activity decreased (Zhou et al., 1995), also inhibited ATP silkworm larvae activity and activity of ATP level in the subcellular distribution dynamics. When fluoride concentration increased, the cylinder-shaped cells of the villi at the ATP directly inhibited the activity decreased, affecting the digestion and absorption of food, high concentrations of fluoride will be fully inhibited the enzyme activity of ATP (Chen et al., 1996). Hemolymph fluoride poisoning of free amino acids increased (Fukami, 1995). Silkworm inorganic fluoride has also hampered the absorption of phosphate ions (Zang et al., 1996). Poisoning in the intestinal wall surface of the silkworm with Mg, P, O and other components of the tiny particles in the cytoplasm of intestinal cells have the same tiny particles, causing K, Mg and P concentrations of regulation and metabolic abnormalities (Fukami, 1995). Under continuous fresh silkworm mulberry leaves treated with fluoride, calcium increased in the skin, reducing the amount of magnesium, two elements that are associated with metabolic abnormalities (Zhou et al., 1998).

The research about pesticides and other hazards of toxic substances

Silkworm is very sensitive to insect pesticides. Pesticide can make silk poising in different ways, such as contact, stomach poison, the smoke, fumigation. Silkworm poisoning phenolmenon is common in present sericultural production, because of the pollution of air and mulberry leaves caused by mulberry field pest control. Variety of pesticides are highly physiologically active substances, their main types are: botanical insecticides, organic pesticides, organic nitrogen pesticides, organochlorine pesticides and so on. Pesticides will inhibit the process of nerve conduction or acetylcholineesterase activity of the normal cycle, so that barriers to silkworm nerve conduction or the performance or the inhibition of abnormal impulses leaving silkworm poisoning; most of organophosphorus pesticides can inhibit the activity of the acetylcholinesterase, which causes acetylcholine cannot break down and formats the accumulation in synaptic release and results in nerve conduction impulses so as to make silkworm poisoning; the organic nitrogen pesticides (such as insecticidal double dimehypo) go into silkworm, acetylcholine as the neurotransmitter, its antagonistic effect will block the nerve impulse conduction, so that it does not generate excitement and make silkworm nerve paralysis. Suzhou College of Sericulture studied the effect on cocooning, cocoon quality and laying eggs with administration trace dimehypo and Chongmanjin drugs for the 5th instar

silkworm, research results showed that if the poisoning took place in the 5th instar silkworm, it will affect cocoon, cocoon quality and spawning (China sericulture Science, 1990). The main pesticides currently on the market, such as fenvalerate (Guo et al., 1991), isofenphos methyl, paraoxon pyrimidine (Dai, 1992), trichlorfon (Dipterex), malathion (Malathion), dipterex2malathion mixture (Zeng, 1994) dimehypo, methamidophos, dichlorvos (Zhiyi and Yuanlin, 1991) quinalphos (Chen, 1995) and other pesticides can cause acute poisoning silkworm. Toxic residue period of commonly used pesticides on the mulberry leaves are different, 1000 times 50% malathion EC is 7 d, 800 times 90% trichlorfon crystal is 10 to 12 d, 500 times 10% quinalphos is 20 d (Zeng, 1994), 1000 times 80% dichlorvos EC is 5 d, 1500 times 50% methamidophos EC is 20 d, 5,000 times 25% bisultap is 60 d, while 10,000 times pyrethroids is over 100 d (Tian et al., 1999). Excessive SO_2 and CO_2 in the air can cause mulberry leaves pollution, though it cannot cause acute poisoning, the health of silkworms was affected and cocoon production and quality will significantly reduce (Zhou and Wensheng, 1991). 1000 mg/kg plant hopper polluted mulberry leaves can cause chronic poisoning (Jiang et al., 1994). Silkworm eats the mulberry leaves with inorganic pollution, such as mercury, arsenic, chromium, lead, silkworm physiques will decline and are vulnerable to the disease (Zhou and Yin 1991).

The research of the prevention and control on non-communicable disease of the silkworm

To prevent fluoride poisoning, silkworm rearing firstly must be layout reasonably, newly-constructing mulberry fields must be far away from the brick, cement plants and other exhaust emissions serious areas and contaminated and nearby school mulberry fields move to the clean area so as not to be polluted. In the main producing areas of sericulture polluted commonly by fluoride, the factories should install the exhausted gas treatment unit shutdown in the silkworm rearing period, establish the monitoring sites for fluoride content in the atmosphere and mulberry leave and strengthen the monitoring of fluoride pollution and timely report test results on fluoride content in mulberry leaves. Fluoride pollution serious areas can choose anti-fluoride silkworm varieties, such as Zhenong 1 × Su 12, Qiufeng × Baiyue, Fengyi × 54A, Xinghang × Keming, Huafeng × Xuesong, 871 × 872 (Zhao et al., 1996; Chen, 1989). The mulberry leaves contaminated by fluorine can be alleviated by water and lime solution washing, then dry the mulberry leaves and feed the silkworm (Lou, 1989). Exhaust emissions by bricks and tiles plants have higher fluoride content; spraying lime solution to line chimneys can reduce fluoride toxic (Zhixian, 1985) or spraying 0.1% aluminum sulfate solution for mulberry polluted leaves has some effect on reducing fluorine content (Wang, 1987); $Ca(NO_3)_2$, $CaCl_2$Ca-EDTA, acetamide, etc., have significant roles in reducing fluoride in the blood and digestive tube, while blood CTS activity decreases, suggesting that it has detoxification effect of different levels and has no significant differences in cocoon characters in comparison to fresh mulberry leaves (Cheng and Zou, 1992). Calcium salts antidote is better than magnesium salts, the detoxification effects of acetamide-CaEDTA and acetamide-CaCl2Ca are better than lime, cocoon weight of the former two antidotes increased by 30 to 35%, cocooning rate index increased by 35 to 40%, close to the fresh mulberry leaves level, while cocoon weight index of lime solution increased only 10%, cocooning rate index increased only 15 (Zouling and Cheng, 1994).

As pesticides for controlling crop pests are toxic to silkworm, therefore, sericultural production areas should plan rationally mulberry area and other crop growing areas and set up rationally sericulture layout; new mulberry fields must be far away from field crops and pollution gas emissions from factories. If mulberry fields have short distance with field crops, field crops such as rice applying pesticides try not to apply poisonous pesticides to silkworm, such as dimephypo and pyrethroid insecticides and granular formulations pesticides so as to avoid contamination of air and mulberry; if silkworm pesticide poisoning occurs, immediately open windows and doors for ventilation in rearing house, timely litter cleaning, and feed with fresh mulberry leaves, in order to save some of the lighter poisoning silkworm, use toxic attachments alkaline wash and expose to strong sunlight. Silkworm poisoning caused by organic nitrogen insecticides, such as dimephypo, can use “Adrenaline hydrochloride” with each (1 ml, content of 1 mg) plus 500 ml of water to add or spray silkworm body or take licorice 100 g, 500 ml of boiling water for half an hour, after cooling, add 100 ml white wine, then add to mulberry leaves or spray silkworm body (Wangliu, 1991; Wan, 2008); organic phosphorus pesticide poisoning can use atropine sulfate (alropine sulfate injection) or 1 or 2 pairs pralidoxime injections adding 500 ml water (Wan, 2008; Qinhuan, 2005) or two pairs pralidoxime chloride injection adding 500 ml water, wet the body or panning silkworm body or use alkaline detergent add water to prepare 100 times solution, fully sprayed on mulberry leaves contaminated with methamidophos, the residue on the leaves, can also be use 30% fresh lime soak 1 h to feed silkworm, which the stated measures can alleviate the toxic effects (Wan, 2008). Atropine Sulfate is widely used in the production, this drug is mainly used in the man and aquatic organophosphorus detoxification as anticholinergics, which through competitive with the M cholinergic receptor binding, the receptor cannot bind with Ach alkali fishy or other drugs with cholinergic, showing the cholinergic nerve is blocked with all the role.

CONCLUSION

This article summarized and reviewed history and progress of China’s silkworm pathological studies. It can be seen that China’s studies on silkworm pathology have achieved great progress. Nowadays, studies on the pathogen *Nosema* have also entered the molecular level. Methodologies for DNA extraction, DNA cloning, DNA sequence analysis, *Nosema* DNA detection by PCR and RAPD markers, *Nosema* small

subunit ribosomal RNA (SSUrRNA) encoding gene and secon-dary structure research had been established. The analyses about microsporidia total protein and surface protein have reached the international advanced level. Silkworm as an important model organism, some researches, however, are currently only at the exploratory stage. Silkworm pathological studies cannot meet the requirements of the basic research. Research aspects such as pathogen's molecular biology, disease-resistant silkworm breeding, pharmaceutical research and development of silkworm disease treatment, non-infectious disease diagnosis and prevention of silkworm and the new pathogen detection methods need further exploration.

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