

Evaluation of anti-diabetic drugs by using silkworm, *Bombyx mori*

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Summary Since diabetes patients are increasing in the world, establishment of a novel method for development of anti-diabetic drugs is desired. In this review, we describe recent results of our studies regarding diabetic model using silkworms for evaluation of anti-diabetic drugs for patients of both type I and type II diabetes. The use of the evaluation systems using silkworms is expected to contribute to reduction in cost and in the number of mammals sacrificed for screening of anti-diabetic drugs.

Keywords: Diabetes, silkworm, *in vivo* evaluation system, anti-diabetic drugs

1. Introduction

Diabetes is a typical life-related disease. Diabetes patients show chronic hyperglycemia, which causes diabetic complications such as retinopathy, nephropathy, and peripheral neuropathy. Diabetes is classified into type I and type II by the difference in mechanism in the onset. Type I diabetes is caused by depletion of insulin, a hormone that regulates glucose level in blood. Type II diabetes is caused by insulin resistance, which occurs either by genetic factors or by life-related diseases such as obesity. In recent years, the number of patients with type II diabetes is increasing in developed countries (1). Insulin and drugs improving insulin resistance are clinically used for diabetes patients. Since side effects such as hypoglycemia and obesity by anti-diabetic drugs take place, the development of new therapeutic drugs, which overcome the problem, is desired (2).

Several hormones such as insulin strictly regulate blood glucose level in mammalian animals including humans. Those hormones adjust uptake, metabolism, and excretion of sugars in the various tissues in the whole body. Therefore, examination with whole animals is needed to evaluate whether candidates of anti-diabetic drugs have an effect on control of blood sugar level. Since sacrificing many mammals cause

problems of high costs and ethical issues in terms of animal welfare, conventional evaluation methods using the mammals such as mice are limited (3). To solve these problems, we tried to develop an invertebrate model with silkworms. We previously reported that the silkworm infection models are useful to quantitatively evaluate the therapeutic effects of antibiotics, anti-fungal drugs, and anti-viral drugs (4-7). Moreover, we revealed common features between mammals and silkworm in the pharmacokinetics of antibiotics and toxic compounds (8). These findings suggested to us that evaluation of therapeutic activities of drugs based on pharmacokinetics using silkworms would be possible. In this review, we introduce our recent findings on the application in the drug discovery by use of hyperglycemic silkworm models.

2. The use of silkworms as model animal for the development of anti-diabetic drugs

Disease models using experimental invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster* are developed (9-14). Since these organisms are very small, it is not easy to measure the sugar level in their hemolymph and to inject compounds with syringe into the bodies of the animals. In contrast, silkworms are relatively big and move slowly. Therefore, injection experiment into the hemolymph of accurate volume of sample solution using a syringe is easy to be performed (15). In addition, silkworm hemolymph in relatively large amounts can be collected. These points are merits of silkworm as compared to *C. elegans* and *D. melanogaster* in the evaluation of anti-diabetic drugs based on the quantitative determination of sugar level

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in the hemolymph. Therefore, we attempted to establish a diabetes model using silkworms and expected that the disease model might contribute to development of anti-diabetic drugs.

3. Evaluation of insulin and AICAR using hyperglycemic silkworms

In a long history of sericulture, mulberry leaves have been used for rearing silkworms. Nutrients contained in the mulberry leaves are absorbed from silkworm intestine to hemolymph and are transferred into the various organs like in mammalian animals (Figure 1A). Silkworms have the organs such as intestine, fat body, and malpighian tubule, which function for exclusion of exogenously administrated chemicals. Moreover, silkworms can maintain glycogen as absorbed carbohydrates in the fat body and the muscle (16,17). Therefore, the systems for uptake of sugars and the storage mechanism show common features between silkworms and mammalian animals including humans.

We considered that silkworms could be used for research regarding diabetes by establishing a technique for measuring sugar level in the hemolymph. As the marked difference in the silkworms and humans, a major sugar in hemolymph of the silkworms is trehalose, which is composed with two molecules of glucose (17). Silkworms synthesize trehalose by the reaction with trehalose synthase in cells of whole bodies and the resulting trehalose is released into the hemolymph (18). Glucose level in the hemolymph of silkworms fed mulberry leaves is very low. Therefore, at the beginning of our study, we wondered whether silkworms become hyperglycemic. In mammals including humans, the blood sugar level is rapidly increased by oral ingestion of sugars such as glucose. We first examined whether glucose level in hemolymph of silkworm would be increased by intake of excess amount of glucose. As a result, we found that total sugar level in hemolymph of silkworms quickly increased within 30 min by intake of an artificial diet containing high amount of glucose (Figure 1B). Moreover, amounts of sugar in fat body, muscle, malpighian tubule, and silk gland in silkworms fed the high glucose diet were much higher than those in silkworm fed a normal diet (Figure 1C) (19). Furthermore, we examined conditions whose silkworms become hyperglycemic by monitoring the effect of the feeding time and the amount of glucose in the diet (Figures 1D and 1F). In addition, glucose by itself was detected in hemolymph of the silkworms by intake of the high glucose diet (Figure 1E). These results suggest that a large amount of glucose is directly transferred from intestinal lumen to hemolymph in silkworms. Based on these findings, we expected that we would be able to establish diabetes models with silkworms, where evaluation of anti-diabetes drugs would be possible.

Next we examined whether hypoglycemic

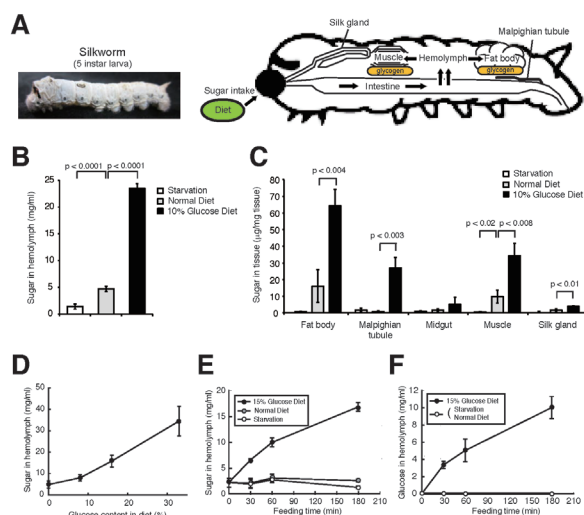


Figure 1. Increase of sugar level in hemolymph and sugar accumulation in various organs of silkworm by intake of high glucose diet. (A) Fifth-instar larvae (left). Transition and distribution in silkworm of dietary sugar (right). (B) Increase of total sugar level in hemolymph of silkworm fed a diet containing glucose (10% glucose diet). (C) Impact on the accumulation of sugar in various organs (fat body, malpighian tubule, midgut, muscle, and silk gland) of silkworm by intake of 10% glucose diet. (D) Effect of glucose content in diet against total sugar level in hemolymph of silkworm. (E, F) Immediate increase of total sugar level (E) and glucose level (F) in hemolymph of silkworm by intake of 15% glucose diet. Statistical significance between groups was evaluated using Student's *t* test. Figures were taken from Matsumoto *et al.* (19) and partly modified.

activities of anti-diabetic drugs could be observed in the hyperglycemic silkworms. The most typical anti-diabetic substance for treatment of patients with type I diabetes is insulin. In mammals including humans, insulin leads to suppression of gluconeogenesis *via* the phosphorylation of Akt in cells of the liver, adipocytes, and skeletal muscle, resulting in stimulation of glucose uptake followed by decrease in blood glucose level (20). Silkworm has bombyxin, a peptide hormone homologous to mammalian insulin. Moreover, the phosphorylation of Akt in the fat body of silkworm was reported to be enhanced by bombyxin (21). We hypothesized that silkworm regulates sugar level in the hemolymph *via* activation of insulin-signaling pathway as in the manner in mammals. To test this notion, we asked whether the hypoglycemic activity of human insulin can be observed in the hyperglycemic silkworms. The result demonstrated that the total sugar level in hemolymph of the hyperglycemic silkworms was decreased by injection of human insulin (Figure 2A). We also found that human insulin enhanced the phosphorylation of Akt in cells of fat body of silkworm in an *in vitro* tissue culture system and stimulated glucose uptake into the fat body (Figure 2B) (19). These effects of human insulin were suppressed by pre-treatment of wortmannin, which is an inhibitor of the phosphoinositide 3-kinase (PI3K), a key factor of insulin-signaling pathway (Figure 2B). These results suggest that human insulin decreases in sugar level of

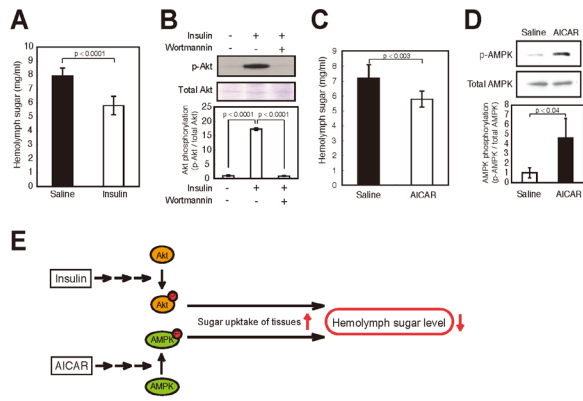


Figure 2. Pharmacological evaluation of human insulin and AICAR by using hyperglycemic silkworm. (A) Decrease of total sugar level in hemolymph of hyperglycemic silkworm by injection of human insulin. (B) Increase of Akt phosphorylation in isolated fat body of silkworm by treatment of human insulin in an *in vitro* culture system and inhibition by wortmannin, an inhibitor of phosphoinositide 3-kinase, against the effect of human insulin. (C) Decrease of total sugar level in hemolymph of hyperglycemic silkworm by injection of AICAR, an activator of AMP kinase. (D) Increase of AMP kinase phosphorylation in isolated fat body of silkworm by treatment of AICAR in an *in vitro* culture system. (E) Mechanism of action in silkworm by administration of human insulin or AICAR. Statistical significance between groups was evaluated using Student's *t* test. Figures were taken from Matsumoto *et al.* (19) and partly modified.

hemolymph in silkworms by enhancing glucose uptake *via* activation of insulin-signaling pathway in cells of tissues including fat body.

In mammals, hypoglycemic effect by activation of AMP kinase (adenosine 5'-monophosphate-activated protein kinase; AMPK) is known as independent system to insulin-signaling pathway. AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) is a compound that activates AMP kinase and promotes to glucose uptake in cells of skeletal muscle in mammals (22). We found that administration of AICAR caused decrease in a sugar level in hemolymph of the hyperglycemic silkworms (Figure 2C). Moreover, the phosphorylation of AMPK in cells of fat body was increased by treatment with AICAR (Figure 2D). These results suggest that AICAR leads to decrease in sugar level in hemolymph of silkworms by activation of AMPK in cells of fat body. Therefore, use of the hyperglycemic silkworms allows us to evaluate anti-diabetic drugs, which activate either insulin-signaling pathway or AMPK pathway (Figure 2E).

4. Evaluation of pioglitazone and metformin using diabetic silkworms

Next, we tested whether hypoglycemic activities of type II diabetes drugs such as pioglitazone and metformin could be evaluated using the hyperglycemic silkworms. The sugar level in hemolymph of the hyperglycemic silkworms, which ate glucose diet for 1 hour, was not decreased by injection of pioglitazone or metformin (23). We considered that the hyperglycemic

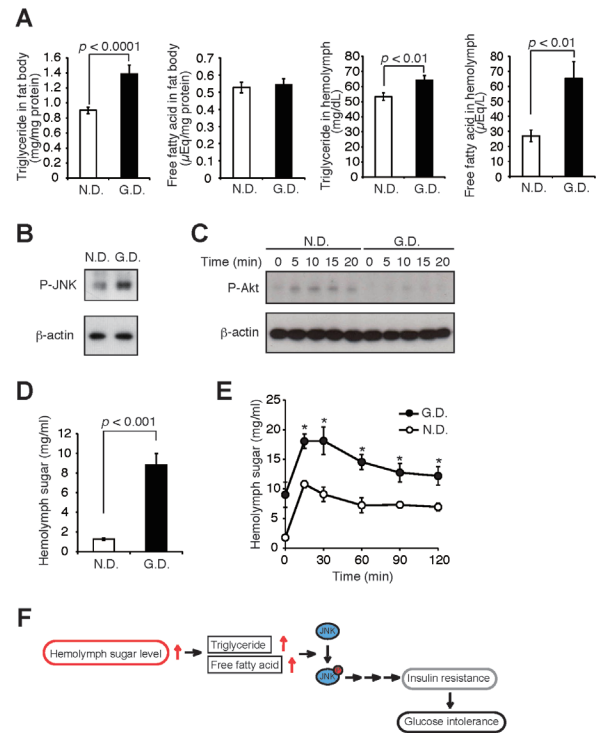


Figure 3. Insulin resistance and glucose intolerance in diabetic silkworm. (A) Accumulation of triglyceride in fat body and increase of triglyceride and free fatty acid in hemolymph of silkworm fed a glucose diet (10% glucose diet) for 18 h. (B) Increase of JNK phosphorylation in fat body of silkworm by intake of 10% glucose diet for 18 h. (C) Insulin resistance in fat body of silkworm by intake of 10% glucose diet for 18 h. The amount of phosphorylated Akt by insulin was determined by Western blot analysis. (D) Increase in fasting sugar level in hemolymph of silkworm by intake of 10% glucose diet for 18 h. (E) Glucose intolerance in silkworm by intake of 10% glucose diet for 18 h. (F) Mechanism of onset of insulin resistance in the diabetic silkworm. N.D.: normal diet. G.D.: glucose diet. Statistical significance between groups was evaluated using Student's *t* test. **p* < 0.05. Figures were taken from Matsumoto *et al.* (23) and partly modified.

silkworms are not mimicked to type II diabetes model, and attempted to establish a new diabetic silkworm model showing symptoms of type II diabetes. We found that the amounts of triglyceride and free fatty acids in hemolymph of silkworms were increased by intake of high glucose diet for a longer period, 18 hours (Figure 3A) (23). Then, we asked whether the hyperlipidemic silkworms under this condition would show symptoms of type II diabetes. Human patients with hyperlipidemia were reported to enhance the phosphorylation of JNK in liver by increase in triglyceride level and free fatty acid level and show insulin resistance (24). We demonstrated that the amount of phosphorylation of JNK in the fat body of the hyperlipidemic silkworms was increased (Figure 3B), and an increase in the phosphorylation of Akt in the fat body by human insulin was suppressed (Figure 3C) (23). Moreover, the hyperlipidemic silkworms showed higher fasting sugar level in the hemolymph and a decrease in glucose tolerance (Figures 3D and 3F) (23). Therefore, we concluded that the

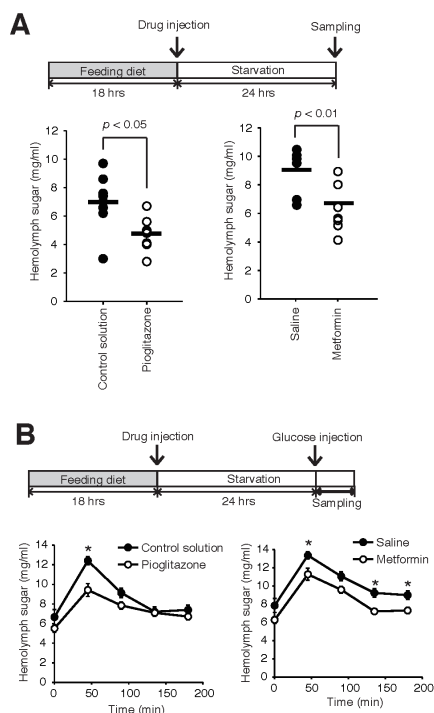


Figure 4. Pharmacological evaluation of pioglitazone and metformin by using diabetic silkworm. (A) Decrease of fasting sugar level in hemolymph of the diabetic silkworm by injection of pioglitazone or metformin. **(B)** Inhibition of decreased glucose tolerance in the diabetic silkworm by injection of pioglitazone or metformin. Statistical significance between groups was evaluated using Student's *t* test. **p* < 0.05. Figures were taken from Matsumoto *et al.* (23).

hyperlipidemic silkworms show the symptoms of type II diabetes.

Next, we tested whether hypoglycemic effects of pioglitazone and metformin can be shown in the diabetic silkworms. As a result, fasting sugar level in hemolymph of the diabetic silkworms was decreased by injection of pioglitazone or metformin, and glucose intolerance was also suppressed (Figure 4) (23). These results suggest that the diabetic silkworms are useful for evaluation of hypoglycemic effects of type II diabetes drugs, such as pioglitazone and metformin.

5. Drug discovery of anti-diabetic drugs using silkworm

Our next challenge in future will be how to screen candidates of anti-diabetic drugs using the diabetic silkworms. Since silkworms are suitable for evaluation of therapeutic effects with a large number of individual animals, the evaluation systems by monitoring the therapeutic effects of candidates using silkworms seem to be useful for following subjects: *i*). Identification and purification of active compounds in natural sources such as herbal medicines and foods, *ii*). Screening of effective compounds from chemical libraries, *iii*). Optimization of active compounds by chemical modifications. Some herbal medicines and food are known to have empirically

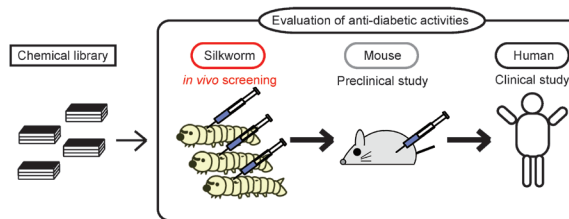


Figure 5. Drug discoveries of anti-diabetic drugs using silkworm.

hypoglycemic effect. However, the active compounds are not yet identified in most cases. Identification by structural determination of the active compounds purified by monitoring the hypoglycemic activities using diabetic silkworms is a conceivable approach. We have reported that polygalactose was identified as an active compound in *Rehmannia Radix* by monitoring the hypoglycemic activities using the hyperglycemic silkworms (19). If large numbers of chemically synthesized compounds are subjected in the evaluation systems using silkworm, it will be possible to select a most active compound having the hypoglycemic activity. The selected compound should be further evaluated in pre-clinical trials using mammalian animal models such as mice, and then would be transferred to clinical trials with human patients (Figure 5). Research using the silkworms is considered to be effective at the first stage before the pre-clinical trials.

6. Conclusion

In this review, we described that the sugar level in hemolymph of the silkworms are increased by intake of high glucose diet. We noted that the silkworm models are useful for evaluation of anti-diabetic drugs for both type I diabetes and type II diabetes. Our study is the first report to propose a screening method of anti-diabetic drugs using invertebrates.

References

1. Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. *Nature*. 2001; 29:782-787.
2. Carver C. Insulin treatment and the problem of weight gain in type 2 diabetes. *Diabetes Educ*. 2006; 32:910-917.
3. Russell WMS, Burch RL. *The Principles of Humane Experimental Technique*. Methuen, London, 1959.
4. Kaito C, Akimitsu N, Watanabe H, Sekimizu K. Silkworm larvae as an animal model of bacterial infection pathogenic to humans. *Microb Pathog*. 2002; 32:183-190.
5. Hamamoto H, Kurokawa K, Kaito C, Kamura K, Manitra Razanajatovo I, Kusuhara H, Santa T, Sekimizu K. Quantitative evaluation of the therapeutic effects of antibiotics using silkworms infected with human pathogenic microorganisms. *Antimicrob Agents Chemother*. 2004; 48:774-779.
6. Matsumoto Y, Miyazaki S, Fukunaga DH, Shimizu K, Kawamoto S, Sekimizu K. Quantitative evaluation of

- cryptococcal pathogenesis and antifungal drugs using a silkworm infection model with *Cryptococcus neoformans*. J Appl Microbiol. 2012; 112:138-146.
7. Orihara Y, Hamamoto H, Kasuga H, Shimada T, Kawaguchi Y, Sekimizu K. A silkworm baculovirus model for assessing the therapeutic effects of antiviral compounds: characterization and application to the isolation of antivirals from traditional medicines. J Gen Virol. 2008; 89:188-194.
 8. Hamamoto H, Tonoike A, Narushima K, Horie R, Sekimizu K. Silkworm as a model animal to evaluate drug candidate toxicity and metabolism. Comp Biochem Physiol C Toxicol Pharmacol. 2009; 149:334-339.
 9. O'Reilly LP, Luke CJ, Perlmutter DH, Silverman GA, Pak SC. *C. elegans* in high-throughput drug discovery. Adv Drug Deliv Rev. 2014; 69-70C:247-253.
 10. Labuschagne CF, Brenkman AB. Current methods in quantifying ROS and oxidative damage in *Caenorhabditis elegans* and other model organism of aging. Ageing Res Rev. 2013; 12:918-930.
 11. Tipping M, Perrimon N. *Drosophila* as a model for context-dependent tumorigenesis. J Cell Physiol. 2014; 229:27-33.
 12. Musselman LP, Fink JL, Narzinski K, Ramachandran PV, Hathiramani SS, Cagan RL, Baranski TJ. A high-sugar diet produces obesity and insulin resistance in wild-type *Drosophila*. Dis Model Mech. 2011; 4:842-849.
 13. Rudrapatna VA, Cagan RL, Das TK. *Drosophila* cancer models. Dev Dyn. 2012; 241:107-118.
 14. Hirabayashi S, Baranski TJ, Cagan RL. Transformed *Drosophila* cells evade diet-mediated insulin resistance through wingless signaling. Cell. 2013; 154:664-675.
 15. Kurokawa K, Kaito C, Sekimizu K. Two-component signaling in the virulence of *Staphylococcus aureus*: A silkworm larvae-pathogenic agent infection model of virulence. Methods Enzymol. 2007; 422:233-244.
 16. Satake S, Kawabe Y, Mizoguchi A. Carbohydrate metabolism during starvation in the silkworm *Bombyx mori*. Arch Insect Biochem Physiol. 2000; 44:90-98.
 17. Horie Y. Blood trehalose and fat-body glycogen in the silkworm, *Bombyx mori*. Nature. 1960; 188:583-584.
 18. Yamashita O, Sumida M, Hasegawa K. Developmental changes in midgut trehalase activity and its localization in the silkworm, *Bombyx mori*. J Insect Physiol. 1974; 20:1079-1085.
 19. Matsumoto Y, Sumiya E, Sugita T, Sekimizu K. An invertebrate hyperglycemic model for the identification of anti-diabetic drugs. PLoS One. 2011; 6:e18292.
 20. Summers SA, Yin VP, Whiteman EL, Garza LA, Cho H, Tuttle RL, Birnbaum MJ. Signaling pathways mediating insulin-stimulated glucose transport. Ann N Y Acad Sci. 1999; 892:169-186.
 21. Nagata S, Hakuno F, Takahashi S, Nagasawa H. Identification of *Bombyx mori* Akt and its phosphorylation by bombyxin stimulation. Comp Biochem Physiol B Biochem Mol Biol. 2008; 151:355-360.
 22. Merrill GF, Kurth EJ, Hardie DG, Winder WW. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am J Physiol. 1997; 273:E1107-E1112.
 23. Matsumoto Y, Ishii M, Hayashi Y, Miyazaki S, Sugita T, Sumiya E, Sekimizu K. Diabetic silkworms for evaluation of therapeutically effective drugs against type II diabetes. Sci Rep. 2015; 5:111180.
 24. Tarantino G, Caputi A. JNKs, insulin resistance and inflammation: A possible link between NAFLD and coronary artery disease. World J Gastroenterol 2011; 17:3785-3794.

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