

A hyperglycemic silkworm model for evaluating hypoglycemic activity of *Rehmanniae Radix*, an herbal medicine

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Summary Silkworm shows hyperglycemia after intake of diet containing large amount of glucose. The hyperglycemic silkworm model is useful for evaluation of anti-diabetic drugs. A hot water extract of *Rehmanniae Radix*, an herbal medicine, showed hypoglycemic effect against the hyperglycemic silkworms. This method is applicable for quick and simple evaluation of the hypoglycemic activities of different batches of *Rehmanniae Radix*. Our findings suggest that silkworms have a lot of merit as experimental animals for evaluation of various herbal medicines.

Keywords: Hyperglycemia, silkworm, *in vivo* evaluation system, *Rehmanniae Radix*, quality check

1. Introduction

Crude extracts of herbal medicines are clinically used without extensive purification of active compounds. Herbal medicines are widely used in Asia. They are also used as a folk medicine in Europe and in America. Therapeutic activity of herbal medicines is known to be greatly affected by their origins and storage conditions, since the amounts of active compounds in herbal medicines are altered by those factors. Therefore, to ensure the maintenance of high quality of herbal medicines, establishment of methods for quality check is needed (1). In general, the quality check of herbal medicine is performed by analytical methods: liquid chromatography-mass spectrometry (LC-MS), fingerprint, quantitative analysis of multi-components by single-marker (QAMS), and thin layer chromatography bio-autographic assay (TLC-BAA) (2). These methods are aimed to identify species and content of secondary metabolites in herbal medicine. However, since active compounds in the herbal medicines are not identified in most cases, there are problems to ensure the qualities of the herbal medicines. To overcome the problem,

establishment of novel quality check methods for monitoring therapeutic activity of the herbal medicines is desired. Previously mammalian animals have been used as model animals for evaluation of therapeutic effect of herbal medicine. However, the use of large numbers of mammalian animals causes serious problems, because it is costly and occurring the ethical issues in terms of animal welfare.

2. Usefulness of silkworms for evaluation of therapeutic effects of herbal medicines

We have proposed that silkworms are useful for evaluation of therapeutic effects and toxicities of drug candidates (3-6). We previously reported that therapeutic effects and pharmacokinetics of chemicals, such as anti-bacterial, anti-fungal, anti-viral drugs, in silkworm were similar to that in mammals (3,4,7-10). Silkworm can be easily handled for evaluation of therapeutic activities of drug candidates using large numbers of individual animals with low costs. Moreover, space needed for rearing silkworms is much smaller than that for mammals. We developed silkworm infection models to understand the molecular mechanisms for pathogenicity of bacteria and fungi (3,10,11). A number of virulence genes in *Staphylococcus aureus* could be identified by large-scale screening (12). The system could be utilized for functional analyses of the virulence genes of *Staphylococcus aureus* (13-17). Accurate volumes of sample solution can be easily administrated into hemolymph of silkworms by injection using a syringe. Such injection experiments are difficult to perform

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with other small invertebrates such as nematodes (*Caenorhabditis elegans*) and fruit flies (*Drosophila melanogaster*). Moreover, biochemical analyses are possible with silkworms, since relatively large amounts of hemolymph can be performed from silkworms. As mentioned above, silkworms have a number of advantages as an experimental animal for the evaluation of therapeutic activity of drug candidates including herbal medicines.

3. Evaluation of the hypoglycemic activities in extracts of *Rehmanniae Radix* using hyperglycemic silkworms

We previously reported that feeding of a high glucose diet causes hyperglycemia in silkworms (18). The hyperglycemic silkworm model is useful for evaluating the hypoglycemic effect of human insulin (18). We also demonstrated that some of the drugs clinically used for diabetes patients showed the hyperglycemic activities in the system. *Rehmanniae Radix* (RehR), a root of *Rehmannia glutinosa* Liboschitz var. *purpurea* Makino or *Rehmannia glutinosa* Liboschitz (Scrophulariaceae), is known to have hypoglycemic activity against mammals, and is widely prescribed for patients with diabetes (19,20). We asked whether the hypoglycemic effect of the RehR could be observed in the hyperglycemic silkworms. Since hot water extract of RehR abundantly contains a large number of monosaccharide such as glucose, sample administration of the hot water extract was expected to cause hyperglycemia in silkworm. Therefore, we tried to purify the substance responsible for the hypoglycemic action in the extract of RehR (Figure 1A). We found that total sugar level in hemolymph of the hyperglycemic silkworms was decreased by injection of the fraction (Figure 1B). We demonstrated that a major component in the active fraction was polygalactose (Figure 1C). Based on these results, we considered that quality check of the RehR is possible to measure the hypoglycemic activity of various batches of RehR using the hyperglycemic silkworms.

4. Establishment of a simple protocol for evaluation of different batches of the RehR using the hyperglycemic silkworms

For the quality check of herbal medicines, the method should be simple so that the results are highly reproducible, and are able to be performed with low costs. We proposed a new method for preparing the active compound in the RehR extracts. In the previous protocol (Figure 1A), five days with five steps were needed (18). Since we previously demonstrated that polygalactose was the active compound in RehR, we tried to establish a new protocol for rapid purification of polysaccharides in the extract of the RehR. From a view of chemical nature of polygalactose, the compound is

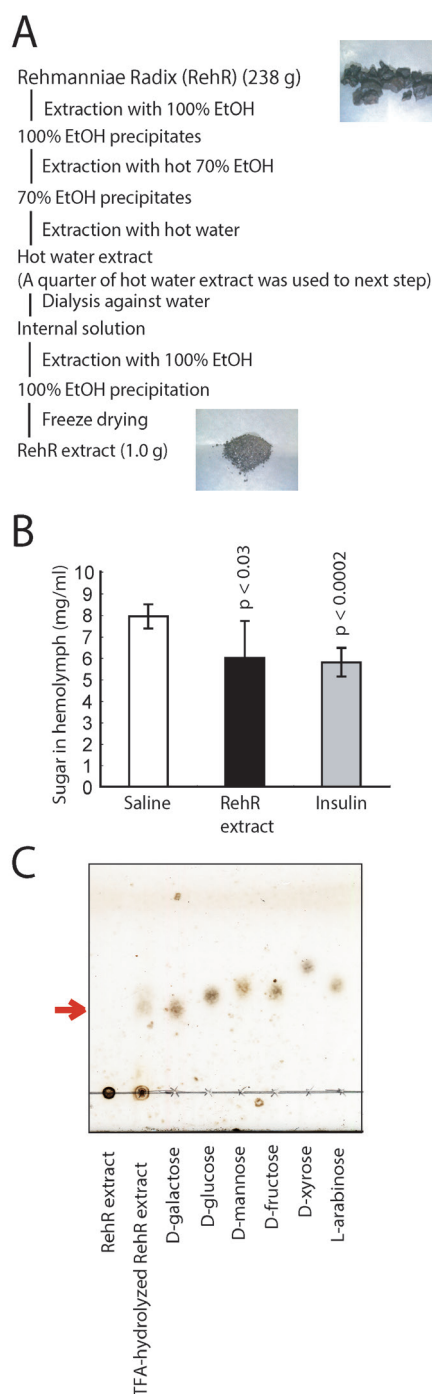


Figure 1. Identification of a hypoglycemic compound in *Rehmanniae Radix* using hyperglycemic silkworm model. (A) Preparation of a *Rehmanniae Radix* (RehR) extract. (B) Decrease of total sugar level in hemolymph of hyperglycemic silkworms by injection of the RehR extract. (C) Analysis of polysaccharides in the RehR extract by thin layer chromatography. Statistical significance between groups was evaluated using Student's *t* test. Figures were taken from Matsumoto *et al.* (18) and partly modified.

expected to be separated from monosaccharides such as glucose by ethanol precipitation. Based on this notion, we established a new protocol, which is shown in Figure 2A (21). According to the new protocol, we obtained RehR extracts (0.2 g) by ethanol precipitation

from hot-water extracts of 6 different batches of the RehR (5 g) (Figure 2A). A control experiment was done with the same batch (#1) of the RehR extract (extract 1), whose fraction according to the previous protocol (Figure 1A) showed the hypoglycemic activity in the hyperglycemic silkworms (Figure 1B). We prepared the purified fraction by the new protocol (Figure 2A), where the number of steps was reduced from 5 steps to 2 steps. Period needed for preparation was reduced from 5 days to 1 day in the new protocol. The final fraction was dissolved in saline at the concentration of 1 mg/mL and was served to examination of hypoglycemic activity in the hyperglycemic silkworms. The sugar

concentration in hemolymph of the hyperglycemic silkworms injected with the purified fraction from batch #1 of the RehR was smaller than that injected with saline, and the difference was statistically significant since the p-value was less than 0.05 by the Student t-test. We next prepared the purified fractions from 5 different batches of the RehR. All of 5 fractions according to the new protocol showed smaller values of the sugar concentration in the hyperglycemic silkworms than the control, however, the differences of extract 4, 5, and 6, were not statistically different since the p-values were larger than 0.05 (Figure 2B). The results suggest that the statistical difference will provide a good evidence

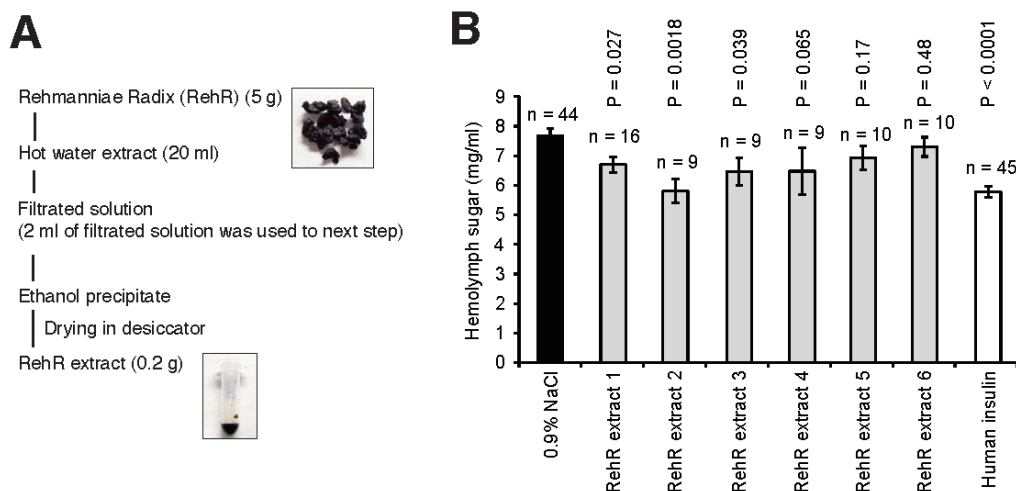


Figure 2. Hypoglycemic activity against hyperglycemic silkworm in different batches of Rehmanniae Radix extracts prepared by a newly established method. (A) Improved method for preparation of Rehmanniae Radix (RehR) extract. **(B)** Decrease of total sugar level in hemolymph of hyperglycemic silkworms by injection of the RehR extracts, which were prepared by the improved method. Statistical significance between groups was evaluated using Student's *t* test. Figures were taken from Matsumoto *et al.* (21).

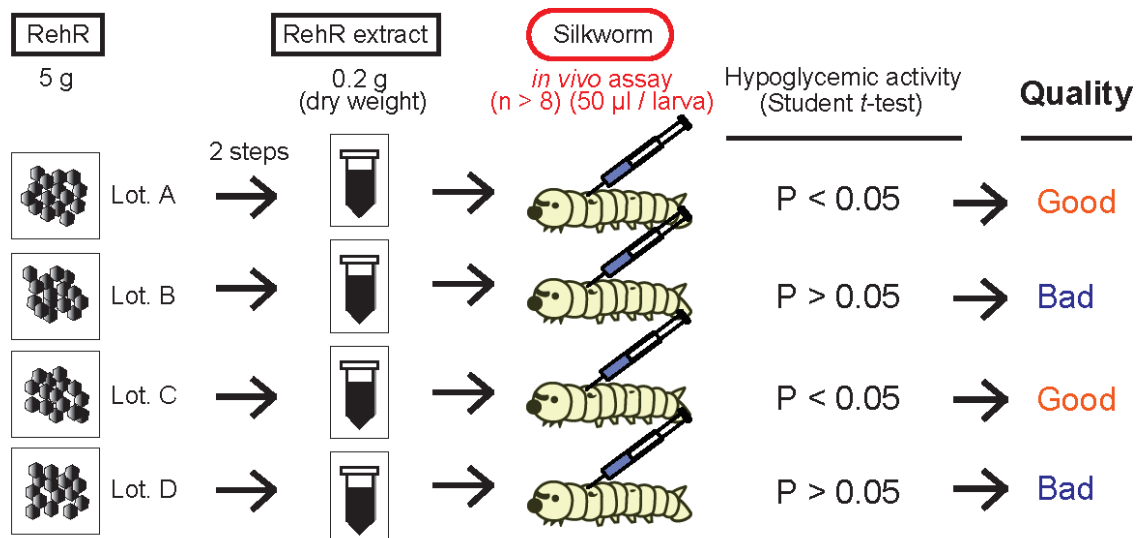


Figure 3. Quality check of Rehmanniae Radix using hyperglycemic silkworms. By comparing total sugar level in hemolymph of hyperglycemic silkworms between sample administration group and saline (control) administration group, $p < 0.05$ in statistically significant difference by Student *t*-test was judged to "good". $p > 0.05$ was judged to "bad".

of RehR for hypoglycemic activity (Figure 3).

5. Versatility of the quality check of herbal medicines using silkworms

For quality check of herbal medicines, mammalian animals have been used for evaluation of efficacies of the herbal medicines. However, mammalian animals such as mice and rats have problems of high breeding costs and ethical issues from the view of animal welfare, it is not suitable for evaluation of large number of the herbal medicines (22). Experiments with mammals must be carried out in accordance to 3R that is an international principle, Replacement (developing alternative methods), Reduction (reducing the number of animals) and Refinement (reduce animal suffering) (23). Silkworm is expected to be useful as an experimental animal for *in vivo* evaluation to ensure the quality of herbal medicines. Use of silkworms as an alternative animal are consistent with the idea of Relative Replacement in the 3R. In other words, the evaluation systems using the silkworm are useful alternative experimental systems under restriction of mammalian animal experiments that causes the ethical issues in development and quality check of herbal medicines.

We previously reported that administration of toxic compounds including bacterial toxins caused death of silkworm, and that the LD₅₀ values, amounts of components needed for 50% lethality of animals, against silkworms were well consistent to that against mammals (4,24). Furthermore, toxicity of compounds at doses smaller than LD₅₀ value can be evaluated by monitoring the ALT activity, a tissue damage marker, in the hemolymph of the silkworm (5). Therefore, not only the efficacy, but also the presence of toxic substances in the herbal medicines can be tested by *in vivo* evaluation systems using silkworms.

6. Conclusion

In this review, we described that the hypoglycemic activity of RehR, an herbal medicine, can be evaluated by using the hyperglycemic silkworms. By using statistical analysis, we can provide evidence that the extract have the activity. We propose that silkworm is useful for the evaluation of efficacy of herbal medicines by use of lifestyle-related disease models such as hyperglycemia. We also propose type II diabetes model and "humanized silkworm" model such as transgenic silkworm expressing human insulin receptor (25,26). We are expecting that the *in vivo* evaluation systems using silkworms will be useful for quality check by monitoring the efficacy of various other herbal medicines than RehR.

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