Evaluation of the Hypoglycemic Effects of the Herbal Medicine Rehmanniae Radix Using a Hyperglycemic Silkworm Model

Yasuhiko Matsumoto and Kazuhisa Sekimizu

Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan

Abstract
We developed a new method for evaluating the hypoglycemic effects of Rehmanniae Radix (RehR), an herbal medicine, by monitoring the decrease in the sugar level of hyperglycemic silkworms. Preparation of a test sample obtained from 5 g of RehR, and quantification of the hypoglycemic effects of the sample using the hyperglycemic silkworm model was completed within 2 days. We further applied the newly established protocol to RehR extracts, and found that three of six samples showed hypoglycemic activity against hyperglycemic silkworms, whereas the other three samples did not. These results suggest that the hyperglycemic silkworm model is a useful alternative animal model to evaluate the hypoglycemic effects of RehR.

Key words: herbal medicine, quality control, silkworm, hyperglycemia, Rehmanniae Radix

Introduction
Herbal medicines, which are crude extracts obtained from natural products, are used for the prevention and treatment of various diseases in Asia, and also as folk medicines in Europe and the Americas (Wills et al., 2000). The medicinal effects of herbal medicines are strongly affected by the production areas and preservation conditions. The amount of active compounds in the herbal medicines may differ between batches. Several analytical methods, such as liquid chromatography-mass spectrometry, fingerprint, quantitative analysis of multi-components by single-marker, and thin layer chromatography bio-autographic assays are used to determine the quality of herbal medicines (Gao et al., 2011). Therapeutic effects, however, cannot be determined by these analytical methods because of the limited number of identified compounds responsible for the therapeutic effects of herbal medicines. Therefore, quality control techniques to insure the therapeutic activities of the herbal medicines by biologic assays are necessary.

Mammalian disease models, such as mice and rats, are generally used to test the therapeutic activities of medicines. Ethical issues, such as animal welfare, and cost, however, limit the use of mammals for quality control of herbal medicines. We previously proposed that silkworm disease models are useful for evaluating the therapeutic activity and toxicity of antibiotics and other drugs (Inagaki et al., 2012; Hamamoto et al., 2009; Kaito et al., 2002; Matsumoto et al., 2012; Orihara et al., 2008). There are many benefits of silkworms as model animals, such as their lower maintenance cost, less space required for keeping the animals, fewer ethical problems compared with mammals, and smaller body weight requiring less drug for evaluation. We previously reported the common chemical pharmacokinetics in silkworms and mammals (Asami et al., 2010; Hamamoto et al., 2004;
Hamamoto et al., 2009). In the Japanese Pharmacopoeia, Rehmanniae Radix (RehR), the root of Rehmannia glutinosa Liboschitz var. purpurea Makino or Rehmannia glutinosa Liboschitz (Scrophulariaceae), with or without steaming is considered to be effective for diabetic patients and is widely used in Asian countries (Kiho et al., 1992; Zhang et al., 2008). The active compound for the hypoglycemic effect in RehR was unknown until recently. We previously established a hyperglycemic silkworm model to evaluate the therapeutic effects of anti-diabetic drugs such as human insulin (Matsumoto et al., 2011). The insulin-signaling pathway, which is essential for maintaining blood glucose levels in mammals, is also present in insects, such as silkworms (Nagata S. et al., 2008). We evaluated the hypoglycemic activity of RehR extract in hyperglycemic silkworms, and determined that poly-galactose was responsible for this activity (Matsumoto et al., 2011). Our findings suggested that hyperglycemic silkworms could be used to evaluate the hypoglycemic effects of herbal medicines like RehR. In the present study, we established a simple preparation method for obtaining an active fraction of RehR and developed a protocol for determining the therapeutic activity by monitoring decreases in sugar levels in hyperglycemic silkworms.

Materials and Methods

Chemicals
Recombinant human insulin and perchloric acid were purchased from Wako (Osaka, Japan). D-Glucose was purchased from Nacalai Tesque (Kyoto, Japan).

Crude drugs
RehR 1 (Uchida Wakanyaku [Lot. SU312910]) was purchased from Uchida Wakanyaku (Tokyo, Japan) and stored in the Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, University of Tokyo. RehR2 (NIB-0155), 3 (NIB-0021), 4 (NIB-0071), 5 (NIB-0045), and 6 NIB-0126 were samples collected from Japanese markets by the Research Center for Medicinal Plant Resources (RCMPR), National Institute of Biomedical Innovation, stored in the RCMPR. All RehR samples were the Japanese Pharmacopoeia crude drugs.

Preparation of Rehmanniae Radix extracts
Five grams of RehR was added to 20 mL MilliQ water, and boiled for 30 min. The hot water extract was passed through filter paper (Whatman™ chromatography paper 3MM Chr, GE Healthcare Japan Corporation, Tokyo, Japan). Two milliliters of the filtered extract was added to 38 mL of ethanol and mixed. The precipitate was collected by centrifugation at 15,000 rpm (20,400 g) for 3 min, and dried in a desiccator (ND-3S, AS ONE, Osaka, Japan) overnight.

Hyperglycemic silkworms
The hyperglycemic silkworm model was constructed according to a previous report (Matsumoto et al., 2011). Silkworms were raised from fertilized eggs to fifth-instar larvae. The fifth-instar larvae were fed a 12%-glucose containing diet for 1 h. Fifty microliters of test sample was injected into the silkworm hemolymph through the dorsal surface using a 27-gauge needle. The injected silkworms were incubated at 27°C without food for 6 h, and hemolymph was collected.

Determination of sugar in the hemolymph
Hemolymph sugar levels were determined by the method described previously (Matsumoto et al., 2011). Fifty microliters of test sample was injected into the silkworm hemolymph through the dorsal surface using a 27-gauge needle. The injected silkworms were incubated at 27°C without food for 6 h, and hemolymph was collected.

Statistical analysis
Data are shown as the mean ± standard error of the mean (SEM). The significance of differences was calculated using a 2-tailed Student’s t-test at the significance level alpha = 0.05.
Results
We previously established a hyperglycemic silkworm model for evaluation of the hypoglycemic activity of drugs. In the present study, we aimed to develop a new experimental protocol for evaluation of the hypoglycemic activity of RehR, an herbal medicine. Because the hot water extract of RehR contains large amounts of sugars such as glucose, injection of the fraction increases the sugar level in the silkworm hemolymph. Therefore, the hot water extract cannot be used to measure the hypoglycemic activity of RehR. We previously reported that poly-galactose isolated from RehR extract has hypoglycemic activity in hyperglycemic silkworms (Matsumoto et al., 2011). Our previous protocol for preparing poly-galactose from RehR included five steps and required a long period of time (5 days; Table 1). To establish a simpler protocol, we first established a quick method of preparing the active compound from RehR based on our findings that the active compound was poly-galactose. Poly-galactose is effectively extracted by hot water, and easily separated from glucose and other sugars by ethanol precipitation. The protocol was designed as shown in Table 1.

According to the new protocol, we prepared an extract from RehR (extract 1), which is the same lot used in our previous report (Fig. 1). We tested whether the extract 1 had hypoglycemic activity in hyperglycemic silkworms. Solution (1 mg/mL) in saline was injected into the hemolymph of the hyperglycemic silkworms. Silkworm hemolymph was collected at 6 h after in-

Table 1 Newly establish protocol for preparation of RehR extracts

<table>
<thead>
<tr>
<th>Step 1</th>
<th>[New protocol] (Total 1 day)</th>
<th>[Previous protocol(^a)] (Total 5 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 2</td>
<td>• Hot water extraction</td>
<td>• Hot 100% EtOH precipitation</td>
</tr>
<tr>
<td>Step 3</td>
<td>• 95% EtOH precipitation</td>
<td>• Hot 70% EtOH precipitation</td>
</tr>
<tr>
<td>Step 4</td>
<td>• Hot water extraction</td>
<td>• Hot water extraction</td>
</tr>
<tr>
<td>Step 5</td>
<td>• Dialysis with water</td>
<td>• 70% EtOH precipitation</td>
</tr>
</tbody>
</table>

\(^a\) Matsumoto et al., 2011

![Figure 1](image.png)

Figure 1 Scheme of a new RehR extract preparation method
Starting with 5 g of RehR, 20 mL of hot water extract was obtained. From one-tenth of the extract, 0.2 g of the final fraction was obtained.
jection, and the amount of sugar was determined by the phenol-sulfuric acid method. The sugar level in the silkworm hemolymph injected with extract 1 was significantly decreased compared to that of the silkworms injected with saline (p = 0.027).

According to our newly established protocol, we further obtained extracts from five other lots of RehR, and measured the hypoglycemic effects in hyperglycemic silkworms. The results demonstrated that sugar levels in the silkworm hemolymph injected with RehR extracts 2 and 3 decreased significantly compared to that of silkworms injected with saline (p < 0.05; Fig. 2). In contrast, hemolymph sugar levels in silkworms injected with RehR extracts 4-6 were not significantly decreased compared to that of silkworms injected with saline (p > 0.05; Fig. 2). The results suggest that the amounts of active compounds in the RehR 4-6 are lower than that in RehR 1-3.

**Discussion**

In this study, we established a new protocol for preparing RehR extract, which has hypoglycemic activity in hyperglycemic silkworms. This new method can be easily performed in a shorter period with much less RehR compared with the previously established method. According to our new protocol, test samples can be prepared within 2 days (Table 1). Moreover, less than 5 g RehR is sufficient in our new protocol. The amount of the starting material is 40 times lower and the operating time is 5 times shorter than those of the previously established method. Furthermore, using the hyperglycemic silkworm model, fewer than 10 h are required to determine hypoglycemic activity in a sample. The preparation of hyperglycemic mice by administration of streptozotocin, which has cytotoxic activity against the beta cells of the pancreas, requires at least 3 days for pharmacological tests (Kumar *et al.*, 2012). The preparation of hyperglycemic silkworms fed a high-glucose diet requires only 1 h (Matsumoto *et al.*, 2011). Therefore, our new method using hyperglycemic silkworms is highly useful for rapid determination of the hypoglycemic activity of RehR. For quality control of herbal medicines, the evaluation method should be rapid, easy, low cost, and reproducible. Our method using silkworms satisfies these requirements.

For evaluations of the therapeutic effects of medicines, mammalian models are commonly used. Experiments using mammals, such as mouse and rat, are problematic from an ethical
perspective of animal welfare. Experiments with model animals should be performed following the 3Rs, i.e., Replacement, Reduction, and Refinement. The 3Rs concept is an internationally recognized principle for responsibly conducting animal experiments (Russell and Burch, 1959). The use of silkworms is consistent with the idea of "Relative Replacement" in the Replacement of the 3Rs. Our findings suggest that the silkworm is a suitable substitute animal model for mammalian models for quality control, based on evaluation of the therapeutic effects of drugs, including herbal medicines.

Evaluations of medicines using silkworms are useful not only for determining the therapeutic effects of drugs, but also for determining toxic contaminants in the fractions. We reported that the LD50 values of various toxic compounds are similar between silkworms and mammals (Hamamoto et al., 2009). Our findings suggest that silkworms are useful for evaluating the toxic effects of contaminants in herbal medicines.

In summary, we propose the usefulness of the silkworm model for evaluating the therapeutic effects of herbal medicines. We expect that in vivo evaluation methods using silkworms will greatly contribute to decrease the number of mammalian models for quality control of herbal medicines.

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References


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Corresponding author:
Kazuhisa Sekimizu, Ph.D.
Laboratory of Microbiology,
Graduate School of Pharmaceutical Sciences,
The University of Tokyo
7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan
Tel.: +81-358-414-820
Fax: +81-356-842-973
E-mail: sekimizu@mol.f.u-tokyo.ac.jp