# Effect of *Gosha-jinki-gan*, a *Kampo* Medicine, on Enhanced Platelet Aggregation in Streptozotocin-Induced Diabetic Rats

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ABSTRACT—We evaluated the effects of *Gosha-jinki-gan* on platelet aggregation in streptozotocin-induced diabetic rats. Enhanced ADP (2 $\mu$M)-induced aggregation of platelets obtained from diabetic rats was inhibited by a single treatment with *Gosha-jinki-gan* (0.3, 1.5 g/kg, p.o.). The anti-platelet aggregatory effect of *Gosha-jinki-gan* (1.5 g/kg, p.o.) was attenuated by simultaneous administration of atropine (1 mg/kg, i.p.) and was abolished by combination of atropine with Hoe 140 (250 $\mu$g/kg $\times$ 2, i.p.), a bradykinin $B_2$ receptor antagonist or L-NAME (10 mg/kg, i.p.), an inhibitor of nitric oxide-synthase. These results suggested that *Gosha-jinki-gan* could improve platelet aggregation in diabetes through increased production of nitric oxide via bradykinin $B_2$-receptors and muscarinic acetylcholine receptors.

*Keywords*: *Gosha-jinki-gan*, Platelet aggregation, Nitric oxide

Abnormalities in the blood or vasculature are important factors in the development of diabetic complications (1–3). Previously, it was experimentally demonstrated that inhibiting both enhanced platelet aggregation and decreased peripheral tissue blood flow are useful in treating diabetic polyneuropathy (4). In recent years, it was suggested clinically that *Gosha-jinki-gan*, which has been used to treating numbness or psychroesthesia in the extremities, low back pain and melosalgia, is especially useful for reducing subjective symptoms in diabetic patients (5). However, there have been few published reports describing basic pharmacological evaluation of the function of *Gosha-jinki-gan*, and the mechanism of clinically observed therapeutic effects has not previously been clarified.

In a previous report, we suggested that the vasodilatory action of *Gosha-jinki-gan*, which was indicated in a clinical study (6), might be a response to increased production of nitric oxide (NO) (7). It was reported that NO induces vasodilation through activation of guanylate cyclase as well as inhibiting platelet aggregation (8). Therefore, in the present study, we evaluated the effects of *Gosha-jinki-gan* on enhanced platelet aggregation and its mechanism in diabetic rats. Furthermore, we also investigated inhibition of platelet aggregation by aqueous extracts of crude components of *Gosha-jinki-gan*.

Male Sprague-Dawley rats were used in all experiments. All animals were given food and drinking water ad libitum, and maintained under a 12-hr cycle of light and darkness. Diabetes was induced by injection of streptozotocin (STZ; 60 mg/kg, i.p.), dissolved in 0.03 M citrate buffer solution (pH 4.5), to 350–410 g rats that had been fasted overnight. Rats were used for experiments 8 weeks after STZ administration, and those showing 450 mg/dl or higher levels of blood sugar were considered to have developed diabetes. Age-matched non-diabetic rats that had received citrated buffer solution (2 ml/kg, i.p.) instead of STZ were used as normal controls.

One hour after oral administration of the test drug or distilled water (10 ml/kg), blood was drawn from the abdominal aorta under ether anesthesia using a syringe containing trisodium citrate (final concentration: 0.313%). Platelet-rich plasma (PRP) was collected by centrifuging the blood specimen at 100 $\times$ *g* for 10 min, and platelet-poor plasma (PPP) was collected after centrifuging the remaining blood at 1,500 $\times$ *g* for 15 min. Using PPP, the number of platelets contained in PRP was adjusted to $3 \times 10^5$ cells/$\mu$l. Maximal platelet aggregation, which was induced by adenosine 5′-diphosphate (ADP) (final concentration: 2 $\mu$M), was determined by the turbidimetric method with an aggregometer (PAM-8C; Mebanix, Tokyo). A 190-$\mu$l aliquot of PRP was put into a small cylindrical cuvette (3121, Mebanix) maintained at 37℃, and 10 $\mu$l of ADP solution was added while stirring with a

magnetic stirrer bar at 800 rpm. The aggregation rate was calculated against the turbidity of PPP, which was designated as 100%. Saline, atropine or $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) was injected i.p. immediately after administration of *Gosha-jinki-gan*, while D-Arg [Hyp$^3$, Thi$^5$, D-Tic$^7$, Oic$^8$] bradykinin (Hoe 140) was twice injected i.p. immediately after and 30 min after the test drug administration. Doses of each crude drug extract were established as equivalent to 1.0 g/kg of *Gosha-jinki-gan* based on the weight-ratio of each crude component in *Gosha-jinki-gan* and the yield of each powder extract.

*Gosha-jinki-gan* was obtained in the form of spray-dried powder from Tsumura (Tokyo). The drug was manufactured from a mixture of 10 crude drugs as described in Table 3. Powder extracts of these crude components were prepared as follows: Twenty-fold water was added to each crude component, and the solution was boiled down to half the previous volume. The condensed solution was centrifuged at $1,500 \times g$ for 5 min, and then the supernatant obtained was freeze-dried. All of these extracts were administered after dissolving or suspending them in water. STZ, sodium ADP, atropine sulfate and L-NAME hydrochloride were purchased from Sigma Chemical (St. Louis, MO, USA). Hoe 140 was purchased from Research Biochemicals International (Natick, MA, USA). Other reagents were purchased from Wako Pure Chemical Industries (Osaka).

The results were expressed as means±S.E.M. Significance was determined by one way analysis of variance (ANOVA) followed by Dunnett's multiple range test. In all cases, $P<0.05$ was considered significant.

In all experiments, there were no significant differences between the groups in body weights or blood sugar levels in diabetic rats (Tables 1–3). The sensitivity of platelets to ADP was significantly increased in diabetic rats as compared to that in non-diabetic controls. Enhanced platelet aggregation in diabetic rats was reduced after administration of *Gosha-jinki-gan* (0.3, 1.5 g/kg, p.o.) (Table 1). The anti-aggregating activity of *Gosha-jinki-gan* (1.5 g/kg, p.o.) was attenuated by simultaneous administration of atropine (1 mg/kg, i.p.) and eliminated by combination with Hoe 140 (250 μg/kg×2, i.p.), a bradykinin $B_2$-receptor antagonist, or L-NAME (10 mg/kg, i.p.), an inhibitor of NO-synthase (Table 2).

Among 10 crude drugs comprising *Gosha-jinki-gan*, only *Alismatis* rhizoma (83 mg/kg, p.o.) and *Dioscoreae* rhizoma (63 mg/kg, p.o.) significantly inhibited ADP-induced platelet aggregation. Anti-platelet aggregation by *Alismatis* rhizoma or *Dioscoreae* rhizoma extracts was antagonized by injection of Hoe 140 (250 μg/kg×2, i.p.) or L-NAME (10 mg/kg, i.p.). Hyperaggregability of platelets was normalized in diabetic rats administered a mixture of *Alismatis* rhizoma extract (83 mg/kg) and *Dioscoreae* rhizoma extract (63 mg/kg) (Table 3).

In the present study, the aggregation of platelets suspended in PPP was investigated determine if the anti-platelet aggregatory effect of *Gosha-jinki-gan* was produced by an NO related product(s) and/or another component(s) such as paeonol (see below) dissolved in the plasma. It has been suggested that plasma factors that inhibit platelet aggregation induced by collagen were present in diabetic rats within 8 weeks after STZ administration (9). Therefore, it was decided that collagen-induced platelet aggregation was not appropriate for evaluating the anti-platelet aggregatory effect of *Gosha-jinki-gan*.

We previously suggested that vasodilation and increased aortic cGMP levels induced by *Gosha-jinki-gan* are due to the activation of NO-synthase because these reactions were abolished by treatment with L-NAME (7). In addition, *Gosha-jinki-gan* did not influence platelet cAMP levels in diabetic rats, but dose-dependently increased cGMP levels (Y. Suzuki et al., unpublished observation). Since improvement of platelet aggregation by *Gosha-jinki-gan* was antagonized by injection of L-NAME, this effect may be the result of increased NO production caused by *Gosha-jinki-gan*. The anti-aggrega-

**Table 1.** Effects of *Gosha-jinki-gan* on ADP-induced platelet aggregation in streptozotocin-induced diabetic rats

| | Non-diabetes | Diabetes | | |
|---|---|---|---|---|
| | | Control | *Gosha-jinki-gan* 0.3 g/kg | *Gosha-jinki-gan* 1.5 g/kg |
| | (N=8) | (N=8) | (N=7) | (N=7) |
| Body weight (g) | 537.0±16.4** | 296.0±17.1 | 309.0±15.2 | 308.2±17.0 |
| Blood sugar (mg/dl) | 133.5±10.1** | 590.6±34.9 | 586.3±25.9 | 609.3±24.7 |
| Platelet aggregation (%) | 36.4±1.9* | 56.9±2.9 | 30.1±6.0** | 25.1±5.0** |

Platelet samples were obtained 1 hr after oral administration of *Gosha-jinki-gan* (0.3, 1.5 g/kg) or distilled water (10 ml/kg). Each value represents a mean±S.E.M. *$P<0.05$, **$P<0.01$, as compared to diabetic control values.

**Table 2.** Influence of atropine, Hoe 140 and L-NAME on anti-platelet aggregatory effect of *Gosha-jinki-gan* in streptozotocin-induced diabetic rats

| | Diabetes | | | |
|---|---|---|---|---|
| | Diabetic control + Saline (N=7) | Diabetic control + Atropine (N=7) | Diabetic control + Hoe 140 (N=7) | Diabetic control + L-NAME (N=7) |
| Body weight (g) | 290.2±15.6 | 298.6±15.3 | 299.1±17.5 | 283.3±11.6 |
| Blood sugar (mg/dl) | 565.3±29.5 | 571.0±20.4 | 565.4±16.8 | 611.9±30.2 |
| Platelet aggregation (%) | 60.0±3.7 | 54.0±4.6 | 58.7±3.9 | 51.1±6.5 |
| | Diabetes | | | |
| | *Gosha-jinki-gan* + Saline (N=8) | *Gosha-jinki-gan* + Atropine (N=8) | *Gosha-jinki-gan* + Hoe 140 (N=8) | *Gosha-jinki-gan* + L-NAME (N=8) |
| Body weight (g) | 284.0±10.6 | 293.2±13.1 | 294.3±16.0 | 280.6±9.8 |
| Blood sugar (mg/dl) | 613.3±40.2 | 561.1±15.9 | 594.0±30.4 | 604.6±25.9 |
| Platelet aggregation (%) | 32.0±2.2** | 47.0±4.1$^{\#}$ | 56.8±2.3$^{\#\#}$ | 54.9±4.9$^{\#\#}$ |

Saline (1 ml/kg, i.p.), atropine (1 mg/kg, i.p.) or L-NAME (10 mg/kg, i.p.) was injected immediately after administration of *Gosha-jinki-gan* (1.5 g/kg, p.o.), while Hoe 140 (250 $\mu$g/kg×2, i.p.) was twice injected immediately after and 30 min after the test drug administration. Each value represents a mean±S.E.M. **$P<0.01$, as compared to the value in the diabetic control group. $^{\#}P<0.05$, $^{\#\#}P<0.01$, as compared to the value in the saline-treated group.

tion activity of *Gosha-jinki-gan* was attenuated by atropine and abolished by Hoe 140. These results suggest that bradykinin $B_2$-receptors and muscarinic acetylcholine receptors mediated the activation of endothelial NO-synthase induced by *Gosha-jinki-gan*. However, since the half-life of NO in vivo is in the order of seconds, the inhibition of platelet aggregation observed in the present ex vivo study could not have been induced by plasma NO itself. Stamler et al. (10, 11) demonstrated that endogenously derived NO was stabilized by reactions with free thiol groups of plasma proteins under physiological conditions, which prolongs its half-life as well as preserving its biological activities. The anti-platelet aggregatory effect of *Gosha-jinki-gan* may be dependent on plasma *S*-nitrosoproteins.

Extracts of *Alismatis* rhizoma and *Dioscoreae* rhizoma, which are contained in *Gosha-jinki-gan*, inhibited platelet aggregation, but this effect was eliminated by combination with Hoe 140 or L-NAME. These findings indicated that the inhibition of platelet aggregation by *Gosha-jinki-gan* was due to these two crude drugs. There have not been any published reports demonstrating the anti-platelet aggregatory effects of either *Alismatis* rhizoma extract or *Dioscoreae* rhizoma extract. Further evaluations are necessary to elucidate which components of these crude drugs induce the anti-platelet aggregation through NO production. On the other hand, Hirai et al. (12) reported that platelet aggregation induced by ADP, epinephrine or collagen is inhibited in platelet samples obtained from healthy volunteers who orally ingested aqueous extracts of *Moutan* cortex (3 g/day), a crude drug component of *Gosha-jinki-gan*, for 7 days. They also suggested that the inhibition of thromboxane production by paeonol, a major component of *Moutan* cortex, played an important role in the anti-aggregatory effect of *Moutan* cortex. In the present study, however, we could not confirm the anti-platelet aggregatory effect of *Moutan* cortex. This might be caused by insufficient doses of test drugs and/or differences between repeated administration and single administration. In fact, it was demonstrated that a high concentration of paeonol was necessary to inhibit the first-phase of ADP-induced platelet aggregation without thromboxane $A_2$ production (12). Therefore, it was considered that the dose of *Moutan* cortex used in this study was insufficient to reduce hyperaggregability of platelets in diabetic rats by single administration.

In conclusion, *Gosha-jinki-gan* inhibited enhanced platelet aggregation in STZ-induced diabetic rats. It was suggested that the anti-platelet aggregatory effect of *Gosha-jinki-gan* was based on increased NO production via bradykinin $B_2$-receptors and muscarinic acetylcholine receptors, and the induction of this pathway was attributed to *Alismatis* rhizoma and *Dioscoreae* rhizoma. The therapeutic effects of *Gosha-jinki-gan* observed in diabetic patients may be partly due to improved microcirculation.

**Table 3.** Effect of aqueous extracts of crude drugs comprised in *Gosha-jinki-gan* on enhanced platelet aggregation in streptozotocin-induced diabetic rats

| | Non-diabetes (N=7) | Diabetes | | | | |
|---|---|---|---|---|---|---|
| | | Control (N=7) | *Rehmanniae* radix 332 mg/kg (N=6) | *Achyranthis* radix 205 mg/kg (N=6) | *Corni* fructus 151 mg/kg (N=6) | *Moutan* cortex 86 mg/kg (N=7) |
| Body weight (g) | 478.4±7.5** | 296.2±8.5 | 284.7±16.3 | 298.4±9.7 | 289.2±15.3 | 299.7±10.1 |
| Blood sugar (mg/dl) | 109.0±6.8** | 601.9±22.0 | 600.3±24.0 | 603.2±20.6 | 605.5±13.9 | 614.9±26.3 |
| Platelet aggregation (%) | 33.9±3.6** | 65.6±2.0 | 61.3±5.5 | 63.5±4.7 | 61.0±4.1 | 56.6±4.7 |

| | Diabetes | | | | | |
|---|---|---|---|---|---|---|
| | *Alismatis* rhizoma 83 mg/kg (N=7) | *Dioscoreae* rhizoma 63 mg/kg (N=7) | *Plantaginis* semen 41 mg/kg (N=6) | Processed *Aconiti* tuber 27 mg/kg (N=6) | *Cinnamomi* cortex 8 mg/kg (N=6) | *Hoelen* 4 mg/kg (N=6) |
| Body weight (g) | 292.4±10.7 | 298.4±12.9 | 298.4±10.6 | 298.2±19.1 | 297.8±19.7 | 294.4±9.6 |
| Blood sugar (mg/dl) | 639.7±39.8 | 644.1±46.2 | 603.2±23.7 | 609.7±28.8 | 583.7±28.6 | 611.7±20.3 |
| Platelet aggregation (%) | 43.3±3.7** | 48.3±5.8* | 62.7±4.5 | 57.7±4.0 | 59.0±4.7 | 62.2±5.8 |

| | Diabetes | | | | |
|---|---|---|---|---|---|
| | *Alismatis* rhizoma + Hoe 140 (N=6) | *Dioscoreae* rhizoma + Hoe 140 (N=6) | *Alismatis* rhizoma + L-NAME (N=6) | *Dioscoreae* rhizoma + L-NAME (N=6) | *Alismatis* rhizoma + *Dioscoreae* rhizoma (N=6) |
| Body weight (g) | 292.4±14.7 | 290.4±11.2 | 297.0±15.2 | 296.4±11.2 | 294.8±8.6 |
| Blood sugar (mg/dl) | 612.7±20.5 | 614.2±40.2 | 602.5±17.8 | 592.3±16.0 | 613.3±30.9 |
| Platelet aggregation (%) | 64.3±4.6## | 64.7±3.5# | 65.0±5.4## | 64.8±3.2# | 29.2±2.4** |

*Gosha-jinki-gan* is composed of 10 crude drugs in fixed proportions: *Rehmanniae* radix (5.0), *Achyranthis* radix (3.0), *Corni* fructus (3.0), *Moutan* cortex (3.0), *Alismatis* rhizoma (3.0), *Dioscoreae* rhizoma (3.0), *Plantaginis* semen (3.0), *Hoelen* (3.0), processed *Aconiti* tuber (1.0) and *Cinnamomi* cortex (1.0). Doses of each crude drug extract was established as equivalent to 1.0 g/kg of *Gosha-jinki-gan* based on the weight-ratio of each crude component in *Gosha-jinki-gan* and the yield of each powder extract. Each value represents the mean±S.E.M. *$P<0.05$, **$P<0.01$, as compared to diabetic control values. #$P<0.05$, ##$P<0.01$, as compared to the corresponding values.

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