High Absorbency and Subchronic Morphologic Effects on the Nasal Epithelium of a Nasal Insulin Powder Dosage Form with a Soybean-derived Sterylglucoside Mixture in Rabbits

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A soybean-derived sterylglucoside mixture (SG) is a potential enhancer of the nasal absorption of insulin. The aim of the study was to examine the absorption of insulin given as the powder dosage form with SG and excipient and to determine the subchronic effects of SG on the morphology of rabbit nasal epithelium. The insulin powder dosage form with SG was administered to the rabbit nasal cavity for five successive days. The average bioavailability and the average pharmacological bioavailability of insulin were about 25.0 and 61.6%, respectively. The nasal mucosa was taken from the nasal cavity and side-effects were investigated using an opticphotolight microscope. The insulin powder dosage form with SG produced no signs of inflammation, erosion or squamous metaplasia. These findings indicate that SG can be considered as a safe and effective enhancer and excipient in the nasal insulin powder dosage form.

Key words insulin; nasal administration; sterylglucoside; powder dosage form; morphologic examination

There has been keen interest in the drug delivery of peptides and proteins via nonparenteral routes in recent years. Nonparenteral routes for insulin delivery have been investigated including the nasal, buccal, pulmonary and transdermal routes. In particular, drug administration by the nasal route has been examined by many investigators who have described the various parameters that affect drug absorption in the nose from various delivery systems and related nasal systems.

In an attempt to increase the bioavailability of insulin after nasal administration, a number of enhancers have been investigated. Among them, sodium taurodihydrofusisolate and didecanoyl-β-α-phosphatidyicholine were found to be effective enhancers for the nasal absorption of insulin although they altered nasal morphology.

Previously we have demonstrated the effect of a soybean-derived sterylglucoside mixture (SG) as an enhancer of the nasal absorption of insulin from a liquid dosage form in rabbits. The bioavailability of insulin was increased 11.6% with SG and was an excellent enhancer in phosphate-buffered saline (PBS) and peanut oil. It was more effective than the well-known absorption enhancer, sodium glycocholate. Since the insulin powder dosage form may be better than the liquid dosage form in terms of insulin stability, the powder dosage form was examined using SG and various excipients.

The precise mechanism of SG action is unclear, but we reported that SG interacts with lipids in the nasal epithelium and does not stimulate the nasal mucosa after a single administration. However, all these findings were from an experiment using a single administration and cannot be applied to clinical situations.

For the clinical use of nasal peptide formulations, it is necessary to examine the morphological effects following continuous absorption and monitor safety. Generally, we have to perform one month and three months toxicity studies. Therefore, in this study, we examined the insulin powder dosage form with SG and excipient, and the effects of continuous nasal administration of the insulin powder dosage form with SG on absorption and nasal mucosa for 5 d as the first step of a chronic application.

MATERIALS AND METHODS

Chemicals Bovine insulin (25.7 IU/mg), porcine insulin (27.8 IU/mg) and mucin (bovine submaxillary glands) were purchased from Sigma Chemical Co. (U.S.A.). Carboxyvinylpolymer (CP) was purchased from Wako Pure Chemical Ind. Ltd. (Japan). Sodium carboxymethylcellulose (CMC-Na) was purchased from Kokusan Chemical Co. (Japan). Hydroxypropylcellulose (HPC) was purchased from Nihonsoda Co. (Japan). Bovine albumin was purchased from Irvine Scientific (U.S.A.). SG was kindly supplied by Ryukakusou Co., Ltd. SG is a mixture of the glucosides of β-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%), and brassicasterol (7.2%). All other chemicals were obtained from commercial sources and were of analytical reagent grade.

Preparation of the Insulin Powder Dosage Forms Freeze-dried insulin mixture was prepared as shown in chart 1; insulin (80 mg) was dissolved in 0.1 mol/l HCl, and 100 ml purified water and 800 mg excipient was added. Insulin solution was adjusted to pH 7.4 with 0.1 mol/l NaOH, and then 2800 mg SG was added. The insulin suspension was freeze-dried and the powder was passed through a 200 mesh sieve (0.772 IU/mg). A physical mixture was prepared: insulin (80 mg), SG (2800 mg) and excipient (800 mg) were mixed in a mortar and this powder was passed through a 200 mesh sieve (0.714 IU/mg). A dose (2.0 IU insulin/kg) consists of 0.22 mg insulin, 7.55 mg SG and 2.2 mg excipient per rabbit (3 kg).

Administration Methods The preparations were administered to female Japanese white rabbits weighing 2.5—3.0 kg (Saitama Experimental Animal Supply Co.). A 10.0 cm polyethylene tube with a diameter of 1.05 mm was fitted to
syringe and inserted into the nose. About 10 mg of the powder dosage form was loaded into the syringe and administered to the left side of the nasal cavity (e.g., 2.0 IU/kg) through the tube. The left side was used for the experiments, and the right side as the control. Spraying was accomplished by attaching a sprayer (rubber bulb with reservoir) as previously reported.13,14 Drug was administered once a day for 5 successive days. Rabbits were fasted on the day before the experiment and fed during the 4 h blood sampling period plus 4 h. Then, they were fasted for the experiment the next day.

**Analysis of Insulin and Glucose** A 1 ml blood sample was collected serially from the ear vein. Plasma was separated by centrifugation for 3 min at 3000 rpm. The plasma glucose was assayed by the glucose-oxidase method using the glucose B-test Wako (Wako Pure Chemicals Ind. Ltd.). The insulin concentration in the plasma was determined by EIA using an insulin assay kit (Dinabot Co., Ltd.).

**Bioavailability** The areas under the insulin concentration-time curve (AUC) and the glucose reduction-time curve (AUG) from 0 to 4 h after administration of insulin were calculated using the trapezoidal method.10 The areas above baseline were not included. The serum glucose level at 0 h was taken as the 100% glucose level. The bioavailability and pharmacological bioavailability were expressed on the basis of AUC and AUG and were calculated according to the following equation in which $AUC_{<h}$ and $AUG_{<h}$ were determined after i.v. administration of 0.5 IU/kg of insulin:

$$bioavailability (F) = \frac{AUC/dose}{AUC_{<h}/dose_{<h}}$$  \hspace{1cm} (1)

$$pharmacological bioavailability (D) = \frac{AUG/dose}{AUG_{<h}/dose_{<h}}$$  \hspace{1cm} (2)

The bioavailability measured by insulin and glucose assays shows a linear relationship, as previously reported.14

**Evaluation of Stimulus on Nasal Mucosa** Rabbits were sacrificed using sodium pentobarbital (Nembutal®, Abbot Laboratories, 25 mg/kg) after sampling following 3 or 5 d administrations. The ventral nasal concha was immediately removed from the anterior nasal cavity, as previously reported,11 and the nasal mucosa was fixed in 10% neutral carbonate-buffered formalin for at least 24 h before routine processing. It was then cut vertically, in 4 mm widths, against the nasal mucosa surface at the central region. Each section was dehydrated using a graded series of increasingly concentrated ethanol solutions and then embedded in paraffin wax.

Tissues were divided into small pieces (about 3 μm in thickness) and stained with hematoxylin and eosin. All sections were examined by an optiphot light microscope (Optiphot, Nikon). The state of the nasal epithelium on the dosed side of the septum was qualitatively compared with the untreated side.

**Statistical Analysis** Data from the animal experiments were compared using analysis of variance and Student's $t$-test. A $p$ value $<0.05$ was considered significant.

**RESULTS**

**Preparation of the Insulin Powder Dosage Form by Freeze-Drying and Physical Mixing** We used two kinds of powder dosage forms, physical mixture and a freeze-dried mixture of insulin with SG (insulin : SG=2:90, by weight). When the insulin powder dosage form as a freeze-dried mixture was administered (10.0 IU/kg), rabbits went into hypoglycemic shock. So, Fig. 1 shows the change in the glucose concentration after administration of insulin powder dosage form prepared by freeze-drying (2.0 IU/kg) or physical mixing (10.0 IU/kg). As a result, when the $D$% for the physically mixed powder was 51.1±12.5%, the reduction in plasma glucose was hardly apparent even on administration of 10.0 IU/kg. On the other hand, the $D$% for freeze-dried mixture powder was 100.7±14.6%. The bioavailability for physically mixed powder and the freeze-dried mixture was 2.67 and 26.3%, respectively. The insulin nasal absorption of the freeze-dried mixture was significantly higher than that of the physically mixed powder ($p<0.01$).

**Effect of Excipient on Absorption** Although SG has been used as an enhancer in PBS and oil,10,11 SG was used as an excipient, and its effect was examined in comparison with CP, HPC, CMC-Na, mucin and albumin. The insulin powder dosage form was prepared at a ratio of insulin : excipient = 2 : 70 : 20, by weight. Figure 2 shows the change in plasma glucose after administration of each formulation to the nasal cavity. The formulation with SG only (insulin : SG=2 : 90, by weight) or CMC-Na showed the peak plasma glucose about 1 h after administration. The formulation with mucin or CP showed a peak at about 2 h. The $D$% was 100.7% for SG.
Table 1. Effects of Various Excipients in a Freeze-Dried Mixture of Insulin, SG and Excipients (2:70:20, 2IU/kg) on Area under the Glucose Reduction–Time Curve (AUG) and the Pharmacological Bioavailability (D%)

<table>
<thead>
<tr>
<th>Excipients</th>
<th>AUG (0-6h)</th>
<th>D% (0-6h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(SG only)</td>
<td>100.7±25.2</td>
<td>26.3±0.3</td>
</tr>
<tr>
<td>CP</td>
<td>143.7±72.9</td>
<td>37.5±0.8</td>
</tr>
<tr>
<td>Mucin</td>
<td>119.2±35.0</td>
<td>31.1±0.4</td>
</tr>
<tr>
<td>HPC</td>
<td>121.9±21.9</td>
<td>31.8±0.2</td>
</tr>
<tr>
<td>CMC-Na</td>
<td>108.9±8.5</td>
<td>28.4±0.1</td>
</tr>
<tr>
<td>Albumin</td>
<td>51.3±34.7</td>
<td>13.4±0.4</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.D. (n=3-4). *p<0.05.

Fig. 2. Effects of Various Excipients in the Freeze-Dried Mixture of Bovine Insulin, SG and Excipients (2:70:20) on the Plasma Glucose after Nasal Administration (2IU/kg).

SG: mixture of insulin and SG (2:90). Each value represents the mean±S.D. (n=3-4), *p<0.05, **p<0.01 versus SG. □, CMC-Na; ○, albumin; ●, SG; ■, CP; ○, mucin; △, HPC.

only, 143.7% for CP, 119.2% for mucin, 121.9% for HPC, 108.9% for CMC-Na and 51.3% for albumin (Table 1). The pharmacological bioavailability (D%) was 26.3% for SG only, 37.5% for CP, 31.1% for mucin, 31.8% for HPC, 28.4% for CMC-Na and 13.4% for albumin. SG as an excipient competed with CP since their F% values were not significantly different. Therefore, SG was shown to be not only an enhancer but also an excipient.

Fig. 3. Plasma Glucose after Nasal Administration of Porcine or Bovine Insulin Powder with SG (Insulin:SG=2:90) Prepared by Freeze-Drying (2IU/kg)

Each value represents the mean±S.D. (n=3). ○, porcine insulin; △, bovine insulin.

Fig. 4. Efficacy of Porcine Insulin Powder with SG (Insulin:SG=2:90) Prepared by Freeze-Drying in a Subchronic Study

Rabbits received drug for 5 successive days (2IU/kg). Data represent blood insulin (a) and blood glucose (b) on day 1, 2, 3, 4, or 5 (mean±S.D.; n=3). □, day 1; ○, day 2; △, day 3; ■, day 4; ●, day 5.

Absorption Efficacy Although we used bovine insulin (Figs. 1 and 2), we had to change it to porcine insulin to measure the insulin concentration using the EIA in-
sulin assay kit. The nasal absorption of both insulins was compared by measuring the reduction in plasma glucose (2.0 IU/kg). There was no difference between them in terms of glucose level (Fig. 3) and, therefore, the porcine insulin was used to measure insulin levels.

In a subchronic study to examine the safety and efficacy of SG in nasal mucosa, rabbits were given the insulin powder dosage form in one side of the nasal cavity, once a day for 5 successive days.

The insulin powder dosage form with SG (insulin: SG = 2:90) prepared by freeze-drying was administered successively to the rabbits (2.0 IU/kg). Figures 4(a) and (b) shows the mean plasma insulin and glucose levels each day. The efficacy of SG in enhancing systemic insulin absorption was reflected by a reduction in a blood glucose. SG caused an increase in plasma insulin from 4.5 μIU/ml at time 0 to 27.5—102.9 μIU/ml at 30 min after administration. Then, insulin disappeared quickly from the plasma. The plasma glucose fell quickly, and the maximum plasma glucose reduction was observed about 2 h after administration. The reduction in plasma glucose lasted for more than 6 h on day 1 and day 5 after successive administrations (data not shown). The insulin and glucose levels on day 3 appeared highest and lowest, respectively, but there was no significant difference compared with other days ($p>0.05$).

### The Histopathological Study

Figures 5(a) and (b) are photographs of the nasal mucosa on the third day of administration, and Figs. 5(c) and (d) are on the 3 d of administration. The untreated side of the nasal cavity was not affected by administering the insulin powder dosage form and was used as the control. There was no damage on the 3 or 5 d for both the control and treated group when the histopathological findings and irritation scores were compared. The insulin powder dosage form with SG seems safe when administered once a day for 5 d.

### DISCUSSION

SG, which is a mixture of glucosides, promoted the systemic absorption of insulin applied nasally to rabbits: insulin was not absorbed from the liquid and powder dosage forms without SG. The absorption of SG has been previously reported for insulin suspensions in PBS (pH 7.4) and peanut oil. The absorption efficiency of insulin increased with the amount of SG in the liquid dosage forms. SG is an effective enhancer and excipient, particularly in the powder dosage form prepared by freeze-drying.

The precise mechanism of SG action as an enhancer is unclear, however, we found that the enhancement was reduced 60 min after administration of SG. A similar finding was re-
Table 2. Effects of Continuous Administration of Insulin Powder (2.0 IU/kg) on the Pharmacological Bioavailability (D%) and the Bioavailability (F%) of Insulin

<table>
<thead>
<tr>
<th>Administration day</th>
<th>D% (0–4 h)</th>
<th>F% (0–4 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>52.9±4.9</td>
<td>31.0±16.4</td>
</tr>
<tr>
<td>2nd</td>
<td>58.8±5.6</td>
<td>16.0±4.8</td>
</tr>
<tr>
<td>3rd</td>
<td>69.2±8.4</td>
<td>43.0±27.3</td>
</tr>
<tr>
<td>4th</td>
<td>67.7±4.1</td>
<td>25.8±15.5</td>
</tr>
<tr>
<td>5th</td>
<td>59.2±5.1</td>
<td>9.2±8.4</td>
</tr>
<tr>
<td>Ave.</td>
<td>61.6±8.1</td>
<td>25.0±18.5</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.D. (n=3).

ported by Pillon et al.\textsuperscript{15} and Arturson et al.\textsuperscript{16} using respectively saponin and chitosan containing insulin in solution after nasal administration. In measuring circular dichroism, SG seems not to interact with insulin as a monomer.\textsuperscript{9} Furthermore, SG appears to interact with lipids in the mucosa\textsuperscript{9} and to reduce the mucosal Ca\textsuperscript{2+} concentration.\textsuperscript{5} Several mechanisms of action of enhancers for peptide drugs have been reported, including action on insulin, protection from peptidase in the nasal mucosa, and interaction with the mucosa. Our findings were consistent with those of Pillon et al.\textsuperscript{15} showing that SG interacts directly with the nasal epithelium. However, no morphological changes were detected.

The potential of SG as an absorption enhancer for the nasal delivery of insulin in humans depends on several factors, including the safety and absorption of the drug during chronic administration.\textsuperscript{15} As far as the nasal mucosa of the control group and the group treated with insulin and SG are concerned, no damage was seen on day 3 or day 5 (Fig. 5). The insulin and glucose levels were almost the same for 5 d after nasal administration, and they also showed similar patterns after administration (Fig. 4).

The average bioavailability was maintained at about 25.0% over the 5 d (Table 2). The highest hypoglycemic effect was shown about 2 h after administration. When the pharmacological bioavailability was calculated using AUC in comparison with 0.5 IU/kg i.v., the average pharmacological bioavailability was about 61.6% for the 5 d.

When the amount of SG in the insulin powder dosage form with excipient was reduced from insul:SG : excipient = 2:90:20 to 2:20:70 in CP, mucin and HPC, the bioavail-

ability fell (data not shown). These findings suggest that SG may have specific physicochemical properties as an excipient compared with other excipients.

SG and insulin caused no visible changes and no morphological changes during the subchronic study.

SG appears to have specific physicochemical properties as an excipient as well as an enhancer. Thus, the insulin powder dosage form with SG seems safe for clinical use.

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REFERENCES