

Nasal Recombinant Hirudin-2 Delivery: Absorption and Its Mechanism *in Vivo* and *in Vitro* Studies

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The objective of this study was to investigate the feasibility of systemic absorption of recombinant hirudin-2 (rHV2) by nasal delivery, and its possible absorption mechanism. The degradation of rHV2 in the nasal tissue homogenate and extracts of mucosae of rabbit, as well as the degradation inhibition of enzyme inhibitor (bacitracin) was evaluated. The bioavailability of rHV2 and the improvement with enhancers, after nasal administration in rats was investigated. For further understanding of the transport and uptake characteristics of rHV2, *in vitro* transport experiment under various conditions using diffusion chamber technique in excised rabbit nasal epithelium was performed. It was found that rHV2 underwent rapid degradation in rabbit nasal homogenate, but it was more stable in the extracts of nasal mucosae surface. Bacitracin was able to inhibit the degradation of rHV2 to certain extent. rHV2 was detected in the rat plasma by chromogenic substrate assay after nasal administration and some enhancers also significantly increased the nasal absorption of rHV2. The transport and uptake of rHV2 across nasal epithelium was concentration-dependent and unsaturated, and was significantly inhibited by low temperature, NaN₃, DNP and colchicines, while was less affected by alteration of transport direction. These results demonstrate that the possible absorption mechanism of rHV2 by nasal mucosa appears to be associated with the endocytosis as well as passive diffusion process.

Key words recombinant hirudin-2 (rHV2); nasal absorption; mechanism; degradation

Recombinant hirudin (rHV), a 65—66 amino acid of acidic polypeptide (molecular weight approximately 6900 Da), is one of the most potent inhibitors of thrombin and has proven to have outstanding anticoagulant and antithrombotic activities.¹⁾ Compared with other anticoagulant agents such as heparin, rHV possesses many advantages on safety, antigenicity and toxicity.²⁾ Now rHV has been using for prophylaxis and treatment of heparin-induced thrombocytopenia (HIT), venous and arterial thrombosis, and shunt thrombosis or treatment of disseminated intravascular coagulation (DIC).^{1,3)} Due to its polypeptide and hydrophilic nature, however, to date, rHV is usually given *via* parenteral injection (i.v. or s.c.) to assure efficient delivery of this drug. Unfortunately, in both cases, due to the inconvenience and associated side effects from injection such as local irritation. Its broader use has been limited by the lack of noninvasive delivery methods for this drug. Thus, the development of a convenient, noninvasive, alternative route of administration for rHV2 with comparable pharmacokinetic performance to parental injection would represent a significant advance in anticoagulant therapy. Compared to other non-injection administrations, such as oral formulation, nasal delivery is a much attractive route for its characteristics of avoiding liver first-pass effect, rapid on set action and a higher bioavailability.^{4,5)} However, little attention has been paid to use nasal route as rHV delivery so far.

Although nasal delivery possesses many advantages, protein and peptide drugs through the nasal route is still considerably less efficient than that of injectable route due mainly to intrinsic poor permeability and metabolism in nasal cavity.⁴⁾ Scientists have made a lot of attempts to investigate the effective and well tolerated methods for improving the absorption of these bioactive peptides and proteins by nasal delivery.⁵⁾ Among these approaches, using absorption enhancers still is the most simple and convenient means.

Since the nasal epithelial cells which are joined by the tight junctions are dominant factor preventing hydrophilic macromolecules from permeability,^{6,7)} it is important to study the uptake and transport mechanisms of these compounds for better understanding nasal permeability barrier, as well as the absorption of the proteins and peptides in nasal and the successful performance of delivery systems. Currently the related studies are still quite a few, in fact for most biologically active proteins and peptides, the absorption mechanisms in the nasal cavity have not been firmly established.

The purpose of present study was to investigate the feasibility by nasal delivery of rHV2, as well as the possible absorption mechanisms of rHV2 across the nasal epithelium.

MATERIALS AND METHODS

Materials Recombinant hirudin (rHV2, rHV-Lys47) was obtained from College of Life Science (Peking University, Beijing, China). The specific activity is 6800 AT-U/mg determined by chromogenic substrate assay. Chromozym TH was from Roche Diagnostics (Mannheim, Germany), while casein, hydroxyl-propyl-beta-cyclodextrin (HP- β -CD), bacitracin and colchicines were purchased from Sigma-Aldrich (Beijing, China). Thrombin (76 U·mg⁻¹) were purchased from Sigma Chemical Co. (U.S.A.), and chitosan (MW 250 kD, degree of deacetylation >85%) was from Yuhuan Ocean Biochemical Co., Ltd. (Zhejiang, China). Sodium azide (NaN₃) and 2,4-dinitrophenol (DNP) were from Beijing Chemical Co. (Beijing, China). Ammonium glycyrrhizinate (AMGZ) was the product of XinJiang TianShan Pharmaceutical Industry Co., Ltd. (Xinjiang, China). All other chemicals were analytical grade.

Animals Male rats (Sprague-Dawley, weighing 300 ± 20 g) and male rabbits (Japanese White, weighing 2.5—3.0 kg) were all obtained from the Experimental Animal Cen-

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ter of Weitonglihua (Beijing, China). All care and management of animals were performed with the approval of Institutional Authority for Laboratory Animal Care.

Preparation of the Homogenate of Nasal Mucosal Tissue Rabbit mucosal tissue homogenate was obtained as the method of Gizurarson *et al.*⁸⁾ Rabbit was euthanized with an injection of Nembutal. The mucosal tissue was carefully removed from underlying cartilage and bone and stored at -70°C . Immediately before the experiment, about 100 mg rabbit mucosal tissue was thawed at room temperature for about 10 min and homogenized for 5 min in 1 ml of Ringer's solution (pH 7.4, containing 125 mM NaCl, 5.0 mM KCl, 10.0 mM NaHCO_3 , 1.2 mM NaH_2PO_4 , 1.4 mM CaCl_2 and 11.0 mM D-glucose) using a glass homogenizer under ice-cold condition. The supernatant fraction was obtained from the homogenate by centrifugation at 4000 *g* at 4°C for 10 min and used directly to study the degradation of rHV2.

Preparation of the Nasal Mucosae Extracts The extracts from mucosal and serosal surface were prepared from the rabbits as described by Aryn *et al.*⁹⁾ After rabbit was sacrificed by above method, mucosae were excised immediately. Then freshly excised rabbit nasal mucosae were mounted on the cell opening (0.50 cm^2) of each pair of valiachien half-cell with the mucosal epithelium facing the donor half-cell. Both half-cell was filled with 3.0 ml of Ringer's solution and extracts were prepared by exposing the mucosal and serosal surfaces individual in Ringer's solution at 37°C for 6 h. Each experiment repeated 3 times. After the mucosal and serosal extracts were collected separately, they were immediately refrigerated and used within 1 h of preparation.

rHV2 Degradation in Nasal Tissue Homogenate and Mucosae Extracts The degradation of rHV2 was determined according to the reported method of Chang.¹⁰⁾ Increasing concentrations of rHV2 (0.05–5 mg/ml) were incubated at 37°C for 5 h in the nasal tissue homogenate or mucosae extracts. In addition, 0.5 mg/ml of rHV2 with inhibitor (0.1% bacitracin) was incubated with the nasal tissue homogenate. Samples were taken before and after 10, 20, 30, 60, 120, 180 and 300 min and at once replaced with an equal volume of the test medium. Further degradation was stopped immediately by adding of 1 M HCl and the rHV2 remained was determined by HPLC method.¹⁰⁾

Nasal Administration Experiments rHV2 and various enhancers were dissolved in saline respectively. rHV2 and enhancer solutions were then pooled in equal volumes to give a final solution of rHV2 at concentration of 3.6% (w/v), with 0.5% chitosan (pH 4.5), 5% HP- β -CD or 1% AMGZ respectively. As a control, rHV2 was directly dissolved in saline at same concentration.

Rat was treated by the method previously described.¹¹⁾ The rats were fasted overnight before the study. Anesthesia was induced intraperitoneally by 40 mg/kg sodium pentobarbital and maintained by additional doses of 15 mg/kg as required. The rats were fixed by their back on board and were surgically prepared by cannulations of the trachea to enable breathing and carotid artery to facilitate blood sample collection, and ligation of the oesophagus to avoid swallowing of samples. A $50\ \mu\text{l}$ dose (6 mg/kg) of the formulations was administered into the left nares by a flexible polyethylene tube attached to a microsyringe. rHV2 solution (1 mg/kg) was subcutaneously administered (s.c.) to determine the relative

bioavailability (Fr). Four hundred microliters of blood samples were withdrawn from the carotid artery into the plastic microfuge tubes at predetermined time (0, 20, 40, 60, 90, 120, 180, 240 min). The blood samples was anticoagulated with 3.8% (w/v) trisodium citrate solution in a ratio of 8.25 : 1.75 (v/v), and plasma was separated after centrifugation for 5 min at 4000 *g*. The concentration of rHV2 in plasma was determined by chromogenic substrate method.¹²⁾

In Vitro Transport Experiments After rabbit was sacrificed according to the previous method, the nasal epithelium was excised immediately as described, and it was washed with the transport medium, ice-cold Ringer's solution.¹³⁾ The nasal epithelium was mounted onto the vertical diffusion chamber with the donor chamber volume of 0.5 ml and receiver chamber volume of 5 ml (effective surface area: 0.28 cm^2). After Ringer's solution and 0.5 ml test samples containing 1.0 mg/ml rHV2 and 10 mg/ml casein with or without inhibitors (1 mM DNP, 0.1 mM colchicines and 10 mM NaN_3) were added to the receiver chamber (serosal side) and the donor chamber (mucosal side) respectively, the epithelium was incubated under bubbling with 95% O_2 , 5% CO_2 for 60 min at 37°C . The experiment was terminated by aspirating the test sample and drawing the serosal solution from receiver chamber. The nasal epithelium was taken off and immersed in 10 ml of ice-cold fresh PBS for 5 min to remove the nonspecifically adsorbed rHV2 on the mucosal surface. To remove the rHV2 bound on the membrane, the nasal epithelium was immersed in 10 ml the ice-cold basic washing solution (0.05 M NaOH/0.5 M NaCl), and the tissue was homogenized and mixed with 4% acetic acid. The mixture was centrifuged at 4000 *g* for 10 min. The rHV2 in the resulting supernatant and in serosal solution was determined by chromogenic assay. In addition, the experiments were performed at 4°C and 37°C respectively, with test samples containing rHV2 of 0.25–3.0 mg/ml for investigating the effects of concentration and temperature on the transport and uptake of rHV2.

Assay Procedures rHV2 analysis in the degradation experiment was performed by a reversed phase HPLC system (HP1100, Agilent, U.S.A.). The column was Zobax C₈ $4.6\text{ mm}\times 15\text{ cm}$, $5\ \mu\text{m}$. Column temperature was 23°C . Flow rate was 1 ml/min. Solvent A was 0.1% trifluoroacetic acid in water. Solvent B was 0.1% trifluoroacetic acid in acetonitrile. The gradient was 10 to 48% solvent B (linear) in 30 min.

Chromogenic substrate assay was carried out as the reported method.¹²⁾ The plasma was incubated with 1 M HCl at 65°C for 5 min to defibrinogenate. The mixture was cooled suddenly and naturalised with 1 M NaOH, then centrifuged at 4000 *g* for 15 min. Supernatant ($100\ \mu\text{l}$) was added to $200\ \mu\text{l}$ of 50 mM Tris buffer (pH 8.3) containing 154 mM NaCl and 0.12 U of thrombin, and incubated at 37°C for 5 min. Afterward, $16\ \mu\text{l}$ of chromozym TH (1.9 mM) was added into the incubation mixture and kept on incubating for another 10 min at 37°C . The reaction is terminated by addition of $200\ \mu\text{l}$ of acetic acid (33%) and then the absorbance of the sample was determined at 405 nm with spectrophotometer (U-2000; Hitachi, Japan).

Data Analysis The pharmacokinetic parameters C_{max} , T_{max} and AUC (the area under the plasma concentration–time curve) were calculated using the PC program DAS 1.0 (Drug Clinical Evaluation Center of Anhui, China). The relative

bioavailability (Fr) of rHV2 after intranasal administration was calculated from the AUC using the value after the s.c. administration.

Statistical Analysis Statistical significance was evaluated by using Student's t test. Difference was considered to be significant for values of $p < 0.05$.

RESULTS

Stability of rHV2 in the Nasal Tissue Homogenate and Mucosae Extracts The degradation profile of rHV2 at various concentrations in the nasal tissue homogenate was shown in Fig. 1. The results indicated that rHV2 was able to be degraded at concentration of 0.05 mg/ml, 0.5 mg/ml and 5 mg/ml with 50.5%, 64.2% and 82.3% of dose remaining after 30 min. Since larger concentration of rHV2 demonstrated a higher percentage of remains, thus, it seemed that in the high concentration range the metabolizing enzymes were saturated.

Figure 2 shown the degrading inhibition of bacitracin in the nasal tissue homogenate compared to degradation in the absence of inhibitor. Bacitracin inhibited the degradation of rHV2 (0.5 mg/ml) with the residual percentage of rHV2 87.3% after 30 min compared to 64.2% without inhibitor.

Degradation of rHV2 in the extracts of rabbit nasal mucosae surface were demonstrated in Table 1. It was indicated that the enzymatic activity in the mucosal extracts was smaller compared with nasal tissue homogenate, and was able to slightly degraded rHV2. The percentage of rHV2 degradation in the extracts of mucosal surface at 0.05 mg/ml and 5 mg/ml after 5 h was almost identical, while the percentage rHV2 found in the extracts of serosal surface after 5 h at above concentration was 5.32% and 3.23%. For higher concentration, there was no significant difference between the extracts of mucosal surface and serosal surface. For lower concentration, the degradation effect of mucosal extract was slightly lower than that of serosal extract.

Nasal Absorption of rHV2 in Rat and Its Improvement

The mean plasma concentrations of rHV2 vs. time after nasal administration to rats with or without various enhancers were shown in Fig. 3, and the pharmacokinetic parameters were given in Table 2. When administered intranasally without enhancers, rHV2 was able to be examined, but with poor absorption of Fr 2.14% compared to a subcutaneous injection. When rHV2 was formulated with 5% HP- β -CD, 0.5% chitosan or 1% AMGZ and administered nasally to anesthetized rats, there were significant increase in plasma rHV2 level and C_{max} compared to rHV2 formulated in saline, indicating that the absorption of biologically active rHV2 was enhanced from the rat nose. The increase in rHV2 absorption was also evident from the increase in $AUC_{0 \rightarrow 240}$ values for formulations containing rHV2 plus enhancers.

When rHV2 was formulated with 5% HP- β -CD, 0.5% chitosan or 1% AMGZ, there were 5.1-, 3.6-, and 3.5-fold increase respectively in $AUC_{0 \rightarrow 240}$ compared to the formula containing rHV2 in saline. The absorption increasing effects of enhancers studied according to $AUC_{0 \rightarrow 240}$ values from the *in vivo* experiments could be ranked as: 5% HP- β -CD > 0.5% chitosan \geq 1% AMGZ > no enhancer.

Characterization of rHV2 Transport and Uptake To elucidate the transcytotic mechanism of rHV2, the effects of

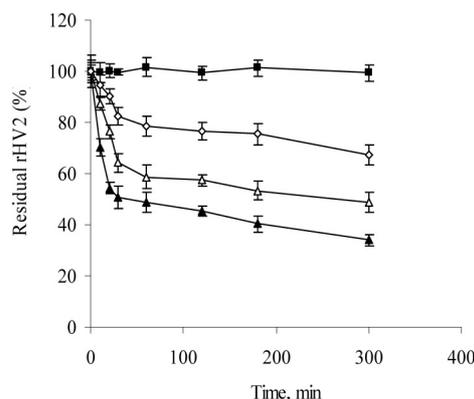


Fig. 1. Degradation of Various Concentration rHV2 in the Nasal Tissue Homogenate of Rabbits

(▲) 0.05 mg/ml; (△) 0.5 mg/ml; (◇) 5.0 mg/ml; (■) 0.5 mg/ml in Ringer's solution. Data were expressed as % of rHV2 remained \pm S.D., $n=3$.

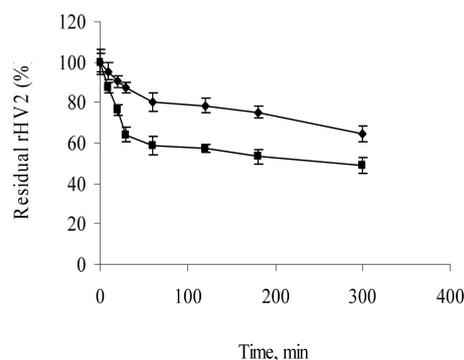


Fig. 2. Degradation of rHV2 (0.5 mg/ml) Alone or with Inhibitor in the Nasal Tissue Homogenate of Rabbits

(■) rHV2, (◆) +1.0% bacitracin; data were expressed as % of rHV2 remained \pm S.D., $n=3$.

Table 1. Degradation of rHV2 (%) after 5 h in the Extracts of Rabbit Nasal Mucosal and Serosal Surface (Mean \pm S.D., $n=3$)

Condition	rHV2 concentration (mg/ml)	Degraded rHV2 (%)
Mucosal surface	0.05	3.16 \pm 0.82
Serosal surface	0.05	5.32 \pm 0.96*
Mucosal surface	5.0	3.01 \pm 0.37
Serosal surface	5.0	3.23 \pm 0.66

* $p < 0.05$ vs. mucosal surface at same concentration of rHV2.

different conditions on the uptake and transport of rHV2 across the excised rabbit nasal epithelium were examined. The results were summarized in Table 3.

Uptake and transport of rHV2 by excised nasal epithelium were concentration-dependent manner at 37°C and 4°C, respectively. When the concentration of rHV2 increased from 0.25 to 3.0 mg/ml (12-fold of initial concentration) at 37°C, saturated absorption was not observed.

The transport of rHV2 from mucosal to the serosal side was significantly inhibited by low temperature, by DNP as an uncoupler of oxidative phosphorylation, by a mitochondrial respiratory inhibitor (NaN_3) and by colchicine as an inhibitor of microtubular assembly, but not by the alteration in transport direction. The amounts in the nasal tissue were also decreased.

DISCUSSION

Recombinant hirudin was a chemically stable polypeptide which thrombin inhibitory activity was maintained even under extreme pH and temperature, as well as digestions with trypsin and chymotrypsin.^{10,14} However recombinant hirudin was subjected to significant degradation in complicated enzyme surrounding, such as, in liver and kidney homogenate, in the luminal contents and subfraction of the intestinal mucosa.^{15,16} There are several proteolytic enzymes on the nasal mucosa surface, in paracellular and in the epithelium cells which could result in the degradation of peptide and protein drugs, such as insulin and human growth

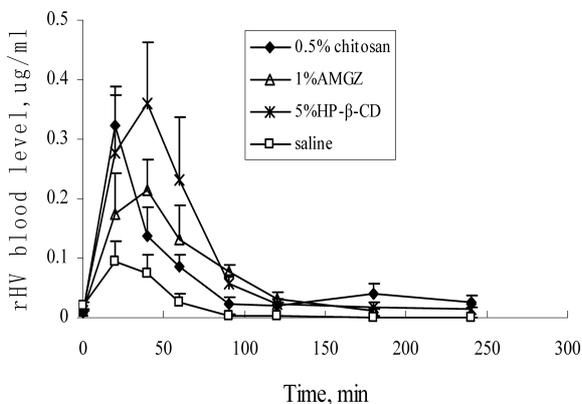


Fig. 3. Plasma Concentration Profiles after Intranasal Administration of rHV2 Solution Alone or with Various Enhancers to Rats

Each value represents the mean \pm S.D., $n=5$.

hormone,^{17,18} although the level of main active enzyme for the degradation of peptide and protein drugs in nasal cavity which has been identified as aminopeptidase¹⁹ is much lower than that in the gastrointestinal tract.⁴ Therefore, to totally understand the degradation of rHV2 in nasal cavity, the extracts of nasal mucosa homogenate and the extract of nasal mucosal surface, which included all kinds of enzyme present in nasal epithelium cell or the extracellular enzymes present on the nasal epithelial surface, were used in our experiment to investigate the degradation of rHV2. It was indicated from the degradation experiments that different concentration of rHV2 was degraded rapidly by nasal mucosa homogenate to 50–82% of the initial concentration of rHV2 within 30 min. The percentage of remain rHV2 decreased below 67% after 5 h. In addition, the degradation of rHV2 was concentration-dependent, larger concentration of rHV2 demonstrated a higher percentage of remains. Thus, it seemed that in the high concentration range the metabolizing enzymes were saturated and the enzymatic degradation appeared to be inhibited by increasing the concentration of rHV2. Bacitracin, an aminopeptidase inhibitor, was able to inhibit this degradation to certain extent, but was not very significant. Whereas rHV2 was more stable in extracts of nasal mucosae surface, suggesting that the enzymatic activity in nasal mucosae extracts was lower than that in nasal tissue homogenate. Thus a formulation designed through paracellular transport pathway of rHV2 for nasal delivery appeared more benefit. In addition, it will be necessary to further isolate and identify the proteolytic enzymes of rHV2 as well as select specific protease inhibitors.

Table 2. Pharmacokinetic Parameters after Nasal Administration of rHV2 Solution Alone or with Some Enhancers to Rats (Mean \pm S.D., $n=5-6$)

Formulations	Dose (mg/kg)	C_{max} (ng/ml)	T_{max} (min)	AUC_{0-240} ($\mu\text{g} \cdot \text{min}/\text{ml}$)	Fr (%)
Chitosan (0.5%)	6.0	322.80 \pm 132.3**	20 \pm 8.4 ^{##}	16.14 \pm 6.40**	7.66
HP- β -CD (5%)	6.0	359.65 \pm 134.9**	40 \pm 24 ^{##}	22.90 \pm 10.21**	10.87
AMGZ (1%)	6.0	215.40 \pm 91.5*	40 \pm 23 ^{##}	15.87 \pm 5.83**	7.53
Saline	6.0	94.15 \pm 42.36	20 \pm 8 ^{##}	4.50 \pm 1.75	2.14
s.c.	1.0	453.90 \pm 95.3	93 \pm 16	35.10 \pm 9.74	100

s.c.: subcutaneous administration. * $p < 0.05$, ** $p < 0.01$ vs. control (saline). ^{##} $p < 0.01$ vs. s.c.

Table 3. Effects of Various Conditions on the Transmucosal Passage of rHV2 in Rabbit Nasal Epithelium *in Vitro* (Mean \pm S.D., $n=5$)

Conditions	rHV2 concentration (mg/ml)	Concentration of inhibitors (mM)	Amount of rHV2 transported in 60 min (μg)	
			Serosal solution	Mucosal tissue
A to B direction None (37 °C)	0.25		1.337 \pm 0.120	0.195 \pm 0.022
	0.5		1.700 \pm 0.205	0.238 \pm 0.013
	1.0		2.784 \pm 0.180	0.304 \pm 0.032
	2.0		3.741 \pm 0.195	0.381 \pm 0.085
	3.0		5.138 \pm 0.476	0.485 \pm 0.062
None (4 °C)	0.5		0.779 \pm 0.068 ^{##}	0.163 \pm 0.028 ^{##}
	1.0		1.333 \pm 0.049 ^{##}	0.188 \pm 0.022 ^{##}
	2.0		1.575 \pm 0.162 ^{##}	0.229 \pm 0.029 ^{##}
+DNP	1.0	1.0	1.566 \pm 0.071**	0.104 \pm 0.010**
+NaN ₃	1.0	10.0	0.984 \pm 0.033**	0.166 \pm 0.012**
+Colchicine	1.0	0.1	1.696 \pm 0.114**	0.115 \pm 0.065**
B to A direction (37 °C)	1.0		2.589 \pm 0.215	0.183 \pm 0.198
B to A direction (4 °C)	1.0		1.298 \pm 0.172	0.125 \pm 0.098

A to B direction: transport from mucosal side to serosal side. B to A direction: transport from serosal side to mucosal side. ** $p < 0.01$ vs. none of 1.0 mg/ml rHV2 at 37 °C; ^{##} $p < 0.01$ vs. same concentration of rHV2 at 37 °C.

Chromogenic substrate assay can demonstrate the absorption of biological active rHV2 into the blood of animal. Although the bioavailability estimated by chromogenic assay, in present studies, was only 2.14% of the subcutaneous injection after nasal administration of 6 mg/kg rHV2 solution without enhancers, rHV2 was able to be determined in the plasma with T_{max} about 20 min. Concerning the nasal delivery associated cell membrane perturbation, 5% HP- β -CD, 0.5% chitosan and 1% AMGZ were used as absorption enhancers to improve the nasal absorption of rHV2, which can effectively enhance the nasal absorption of peptide and protein, while have less irritant or disruptive effect on the cell membrane.^{20,21)} In the present experiment, when rHV2 was formulated with 5% HP- β -CD, 0.5% chitosan or 1% AMGZ and administered nasally to anesthetized rats, there was a remarked increase in the absorption compared to rHV2 in saline ($p < 0.01$), indicating that biological active rHV2 was significantly absorbed from the rat nose. The enhancing mechanism of cyclodextrins was likely due to the disaggregation of protein aggregates and inhibiting of enzyme activity (insulin), or an interaction with lipids and divalent cation on membrane surface or direct effect on the paracellular pathway by a transient effect on tight junctions.²⁰⁾ Chitosan is a polysaccharide comprised of mainly glucosamine. It exerts its promoting absorption effects on the hydrophilic molecules by its bioadhesion and a transient opening of the tight junction in the cell membrane²²⁾ while the absorption-enhancing activity of dipotassium glycyrrhizinate involves structural alterations in the cytoskeletal actin filaments which are provoked by changes in intracellular calcium ion levels.²³⁾ So, HP- β -CD, chitosan and AMGZ may enhance the nasal absorption of rHV2 by those mechanisms, at same time it also indicated the mucosa transport may be one of the dominant reasons of protecting rHV2 nasal absorption. Therefore further studies were necessary to fully clarify the enhancing mechanism and the perturbation of these formulations in nasal mucosa. In addition, from the T_{max} value of all nasal formulations studies, we also found a rapid onset effect of nasal administration compared to subcutaneous injection. The T_{max} was about 20–40 min after intranasal administration compared to 93 min of subcutaneous injection.

Because the aim of *in vivo* experiment was to confirm the effect of enhancers on the absorption of rHV2 by nasal delivery, as well as the rat nasal epithelium could not provide enough area to fit the device *in vitro* experiment, so we chose rabbit nasal epithelium *in vitro* experiments instead of rat's.

In our *in vitro* experiment, as demonstrated using excised nasal epithelium, rHV2 caused a concentration-dependent increase in uptake and transport across nasal epithelium. Further studies proved that the rHV2 transport was able to be significantly inhibited by low temperature and metabolic inhibitors which can damage the functions of epithelial cell and inhibit the transport of rHV2 through transcellular way such as 1 mM DNP (an uncoupler of oxidative phosphorylation by blocking the conversion of ATP substrates to ATP) and 10 mM NaN_3 (a mitochondrial respiratory inhibitor), as well as 0.1 mM colchicine (an inhibitor of microtubular assembly), suggesting that the rHV2 transport was energy-dependent and ATP production *via* oxidative phosphorylation was essential for rHV2 transport. So the transport route of rHV2 was likely related to the transcellular way. However,

we did not observe rHV2 saturable process, and also no obvious difference in transport direction of rHV2 uptake in the present studies, thus another transport pathway, a passive diffusion would be involved at the same time. Therefore it is concluded from all the findings above that a transcellular transport which requires energy for its operation in the physiological state and unsaturated of concentration exists in the transport process of rHV2, so an endocytotic pathway as well as a passive diffusion process was possible to contribute to the transport of rHV2 through nasal epithelium.

CONCLUSION

rHV2 underwent rapid degradation in rabbit nasal homogenate, but it was relatively stable in the extracts of nasal mucosa surface. Bacitracin was able to inhibit the degradation of rHV2 in rabbit nasal homogenate to certain extent. rHV2 was determined in the rat plasma after nasal administration and some enhancers were able to effectively increase the nasal absorption of rHV2. The absorption mechanism of rHV2 by nasal mucosa appears to be associated with the endocytosis and passive diffusion process.

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