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Amphipathic KALA fusogenic peptide enhances absorption of insulin and calcitonin by pulmonary membranes of rats

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ABSTRACT. – OBJECTIVE: The aim was to investigate the absorption-enhancing effect (AEE) of lysine-alanine-leucine-alanine (KALA) repeating unit peptide upon pulmonary absorption of peptide and protein medicines among rats.

MATERIALS AND METHODS: Absorption of insulin and calcitonin in the lung was evaluated using varying concentrations of KALA peptide from 0.1% to 1.0% (w/v). The study also examined the lung damage caused by the KALA peptide.

RESULTS: KALA peptide with various concentrations improved the absorption of insulin and calcitonin in the lungs. It also reduced glucose and calcium levels in the blood compared to the control, with the AEE increasing in a concentration-dependent manner due to the KALA peptide. In toxicity assays, test results for protein and lactate dehydrogenase (LDH) in bronchoalveolar lavage fluid (BALF) did not show a significant increase in the presence of KALA peptide at various concentrations. This implies that the KALA peptide did not cause any membrane damage to lung tissues. In transmembrane electrical resistance (TEER) and permeability detection, a decrease in TEER value and an increase in papp value by the addition of KALA peptide indicated that KALA peptide had the ability to aid the drug delivery through epithelial cells *via* both paracellular and transcellular pathways.

CONCLUSIONS: KALA peptides are suitable as an absorption enhancer at lower concentrations (below 1.0%, w/v) for improving the absorption of insulin and calcitonin from the lung with no observed toxic impact.

Key Words:
Kala peptide, Absorption, Lung, Toxicity, Insulin, Calcitonin.

Introduction

The pulmonary route has attracted widespread attention as an effective drug delivery route. Big alveolar surface area, thin epithelial barrier, extensive vascularization, and low enzymatic activity compared to other administration routes make the lungs a suitable portal for the absorption of macromolecular medicines, including peptides and proteins[1-4]. A significant challenge in pulmonary drug delivery is that large molecular medicines like peptides and proteins cannot easily pass through mucosal barriers, which include the epithelial cells of the alveolus, the blood-air barrier, and macrophages. This is because these medicines are hydrophilic and have high molecular weights[5,6]. Because of these absorption barriers, the bioavailability of these drugs from the pulmonary route is still poor compared with the injection route. In order to overcome this problem, many possible strategies have been applied to improve the absorption of peptides and proteins by pulmonary membranes, including various absorption enhancers[7-12] and some carriers such as dendrimers[13], micelles[14], liposomes[15], nanoparticles[16], cyclodextrins[17,18], and so on. Although these strategies achieve lung mucosal absorption of protein and peptide, their bioavailability is still poor compared with the injection. Therefore, it is crucial to develop an efficient and safe absorption enhancer to improve the delivery of protein and peptides from the lungs.

KALA is one kind of basic amphipathic peptide (lysine-alanine-leucine-alanine repeating unit) with an R-helix conformation; one face shows hydrophobic leucine residues, whereas the other face shows hydrophilic lysine residues[19,20]. Positive KALA charges were considered to be able to disrupt mucosal lipid[21-23]. KALA can bind to plasmid DNA to improve its transfection efficiency into various cellular lines; the predominant role of KALA is to promote cellular entry of DNA by offering a fusogenic ability[24-26]. The pur-

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pose of the current study was to investigate the KALA fusogenic peptide on pulmonary absorption of insulin and calcitonin. Apart from insulin, calcitonin was opted for as a model of peptide and protein medicines, and the effects of KALA fusogenic peptide with various concentrations upon the absorption of insulin and calcitonin by pulmonary membranes were examined among rats. Additionally, the effects of KALA fusogenic peptide on pulmonary membrane damage were studied systematically by assessing the protein content and lactate dehydrogenase (LDH) activities in bronchoalveolar lavage fluid (BALF). Transepithelial electrical resistance (TEER) and KALA peptide permeability were also detected to clarify the mechanisms of absorption-enhancing for the KALA peptide.

Materials and Methods

Materials

Fluorescein isothiocyanate-labeled dextrans (FDs) with various molecular weights (FD4, 4,400; FD10, 9,300; FD70, 69,000) were obtained from Novo Biotechnology Co., Ltd. (Beijing, China). 5(6)-Carboxyfluorescein (CF, MW 376) was supplied by Ruinuode Biotechnology Co., Ltd. (Suzhou, China). Insulin (MW 5,807) was purchased by Novo Nordisk Biopharmaceutical Co. (Copenhagen, Denmark). Calcitonin (MW 3,432) was purchased by Beinuo Biotechnology Co., Ltd (Shanghai, China). Sodium pentobarbital was obtained by Ruiyang Chemical Co., Ltd. (Wuhan, China). The whole other reagents and solvents were of analytical rank.

Preparation of Drug Solution

Before the absorption experiment, a predetermined amount of CF, FD4, FD10, FD70, insulin, and calcitonin was dissolved in an isotonic phosphate buffer solution (PBS, pH 7.4). Various concentrations (0.1%, 0.5%, and 5% w/v) of KALA peptide were complemented to different medicine solutions separately as absorption enhancers. The medicine concentrations and doses administered are listed in Table I.

Animal Experiments

All animal experiments were carried out according to the protocol approved by the Animal Ethics Committee at Guili Medical University. Pulmonary absorption experiments were accomplished using the method previously described[10,27]. Briefly, male Wistar rats (220-250 g) were fasted for 12 hours before the experiments. An intraperitoneal injection of 50 mg/kg of sodium pentobarbital was used to anesthetize the animals. The rats were tied to one anatomical plate at 37°C, and the trachea was disclosed by means of a longitudinal incision.

Table I. Dosing regimen of these poorly absorbable medicines with various concentrations of KALA peptide administrated *via* the lungs of rats.

Group of animals	Medicine solutions (PBS pH 7.4)	Medicine concentrations	Doses administered
1	FD4	2 mg/mL	100 µL
2	FD4+1.0% (w/v) KALA	2 mg/mL	100 µL
3	FD4+0.5% (w/v) KALA	2 mg/mL	100 µL
4	FD4+0.1% (w/v) KALA	2 mg/mL	100 µL
5	FD10	2 mg/mL	100 µL
6	FD10+1.0% (w/v) KALA	2 mg/mL	100 µL
7	FD10+0.5% (w/v) KALA	2 mg/mL	100 µL
8	FD10+0.1% (w/v) KALA	2 mg/mL	100 µL
9	FD70	2 mg/mL	100 µL
10	FD70+1.0% (w/v) KALA	2 mg/mL	100 µL
11	FD70+0.5% (w/v) KALA	2 mg/mL	100 µL
12	FD70+0.1% (w/v) KALA	2 mg/mL	100 µL
13	Insulin	10 IU/mL	100 µL
14	Insulin+1.0% (w/v) KALA	10 IU/mL	100 µL
15	Insulin+0.5% (w/v) KALA	10 IU/mL	100 µL
16	Insulin+0.1% (w/v) KALA	10 IU/mL	100 µL
17	Calcitonin	10 µg/mL	100 µL
18	Calcitonin+0.1% (w/v) KALA	10 µg/mL	100 µL
19	Calcitonin+0.5% (w/v) KALA	10 µg/mL	100 µL
20	Calcitonin+1.0% (w/v) KALA	10 µg/mL	100 µL

Lysine-alanine-leucine-alanine (KALA), phosphate buffer solution (PBS), fluorescein isothiocyanate-labeled dextrans (FDs).

Next, the trachea was partially cut transversely between the fourth and fifth rings. A section of 2.5 cm polyethylene tubing was inserted through the tracheal incision for 0.6 cm. About 100 microliters of various medicine solutions were injected into the lungs through an accurate 250 μL syringe (Hamilton®, Hamilton Co., Shanghai, China). 0.2 mL of blood sample was gathered from the jugular vein at a preset time for 360 min.

In certain experiments, insulin, and calcitonin solution in PBS was intravenously administered into the caudal vein by bolus injection in order to calculate the pharmacological availability.

Analytical Methods

The blood concentrations of FD10, FD4, and FD70 were examined with a fluorescence spectrometer (Waters2475, Milford, CT, USA). The plasma concentrations of insulin were determined by means of an insulin EIA Kit (Zike Biotechnology Co. Ltd., Shenzhen, China). Plasma glucose concentrations were also examined using the Glucose Assay Kit (Zike Biotechnology Co., Shenzhen, China). This area under the curve (AUC) was counted by the trapezoidal approach, and the absorption enhancement ratios of medicines in the presence of KALA peptide with various concentrations were figured out by means of the equation:

$$\text{Enhancement Ratio} = \frac{AUC_{\text{with enhancer}}}{AUC_{\text{control (without enhancer)}}}$$

As for calcitonin, plasma calcium levels were examined by means of the Calcium E Test (Beyotime Biotechnology Co. Ltd., Shanghai, China). The decrement of plasma glucose and calcium level (D%) after administrating insulin and calcitonin with or without KALA peptide was calculated by this formula below:

$$D\% = \left(1 - \frac{AUC_{0 \to 360}}{100\%} \times 360\text{min}\right) \times 100$$

The area above the 100% line in the equation was ruled out for calculating the $AUC_{0\text{-}360}$. The pharmacological availability (PA%) was figured out according to the formula below[10,28]:

$$PA\% = \left(\frac{D_{(ip)}\%}{D_{(iv)}\%} \times \frac{Dose_{(iv)}}{Dose_{(ip)}}\right) \times 100$$

Assessment of Membrane Toxicity

The solutions of KALA peptide at different concentrations were perfused to the trachea of rats anesthetized by the isoflurane (ca. 0.1%) inhalation following the previous method[13,28]. The bronchoalveolar lavage fluid BALF was gathered and centrifuged after 6 hours. The protein was examined by means of one protein assay kit (Zike Biotechnology Co. Ltd., Shenzhen, China) using bovine serum albumin (BSA) as a standard. Lactate dehydrogenase (LDH) activity was also examined using an LDH ELISA Kit (Zike Biotechnology Co. Ltd., Shenzhen, China).

Electrophysiological Parameters of KALA Peptide

The pulmonary membranes used in these experiments were obtained from female South African clawed frogs (Xenopus laevis) 50-60 g, as described previously[22]. After fixing the tissue in the diffusion chamber, the KALA peptide solution was poured into the mucosal side, and then an equal volume of PBS (pH 7.4) was added to the serosal side. The tests were conducted under 95% O_2 and 5% CO_2 at 37°C. Short circuit current (Isc) and transepithelial potential difference (PD) were determined at the preset time. Ohm's law was used to calculate the transmembrane electrical resistance (TEER) value.

Permeability of KALA peptide

After the mucosa was fixed in the diffusion cell, the CF solution (0.1 mM) with KALA peptide (0.1, 0.5%, and 1%, w/v) was poured into the mucosal side. Ringer's solution was also complemented on the other side.

A spectrofluorometer was employed to measure the concentration of CF at the predetermined time points, and the apparent permeability coefficient (p_{app}) was calculated using the formula provided below..

$$P_{app} = \frac{dXR}{dt} \times \frac{1}{A} \times \frac{1}{C_0}$$

where C_0 means the primary concentration (μM/ml), A means the diffusion area (0.3026 cm^2), and XR means the quantity of CF (μM/min).

Statistical Analysis

The results are expressed as the mean ± S.E. of three to five animals and statistical significance was

performed by one-way analysis of variances (ANOVA) with $p < 0.05$ as the minimum level of significance. Computer Origin 6.0 software (Northampton, MA, USA) was used for data analysis.

Results

Effect of KALA Peptide Upon the Absorption of Poorly Absorbable Medicines by Pulmonary Membrane

Figure 1 shows drug time curves of FD4, FD10, FD70, and insulin after administrating rats with KALA peptide at various concentrations. The data indicated that KALA peptide with various concentrations from 0.1 to 1.0% w/v markedly enhanced the absorption of FD4, FD10, FD70, and insulin by pulmonary membrane contrasted with control. For poorly absorbable model drugs, the order of absorption-enhancing effect (AEE) was 1% > 0.5% > 0.1% (w/v) for the KALA peptide, indicating a concentration-dependent AEE.

Table II summarizes the AUC values of poorly absorbable medicines and their absorption enhancing rate following the administration with or without various concentrations of KALA peptide. As shown in Table II, various concentrations of KALA peptide significantly enhanced the area below the curve (AUC) of FD4, FD10, FD70, and insulin in contrast to the control, suggesting that KALA peptide was able to increase the absorption of these poorly absorbable medicines from lungs. The absorption enhancement ratio of KALA peptide at the highest concentration (1.0%, w/v) was 4.2 for FD4, 2.7 for FD10, 1.7 for FD70, and 4.8 for insulin, respectively.

Figure 2 showed concentrations-time curves of glucose and calcitonin in the blood after administrating insulin and calcitonin to rats with KALA peptide at varying concentrations. As shown in Figure 2, we observed a significant decrease in plasma glucose levels after administering insulin combined with varying concentrations of KALA peptide to rats. Besides, one similar consequence was observed in the case of calcium levels in plasma (Figure 2). Table III summarizes pharmacodynamic parameters (D%, PA%, along with enhancement rates) of insulin and calcitonin be-

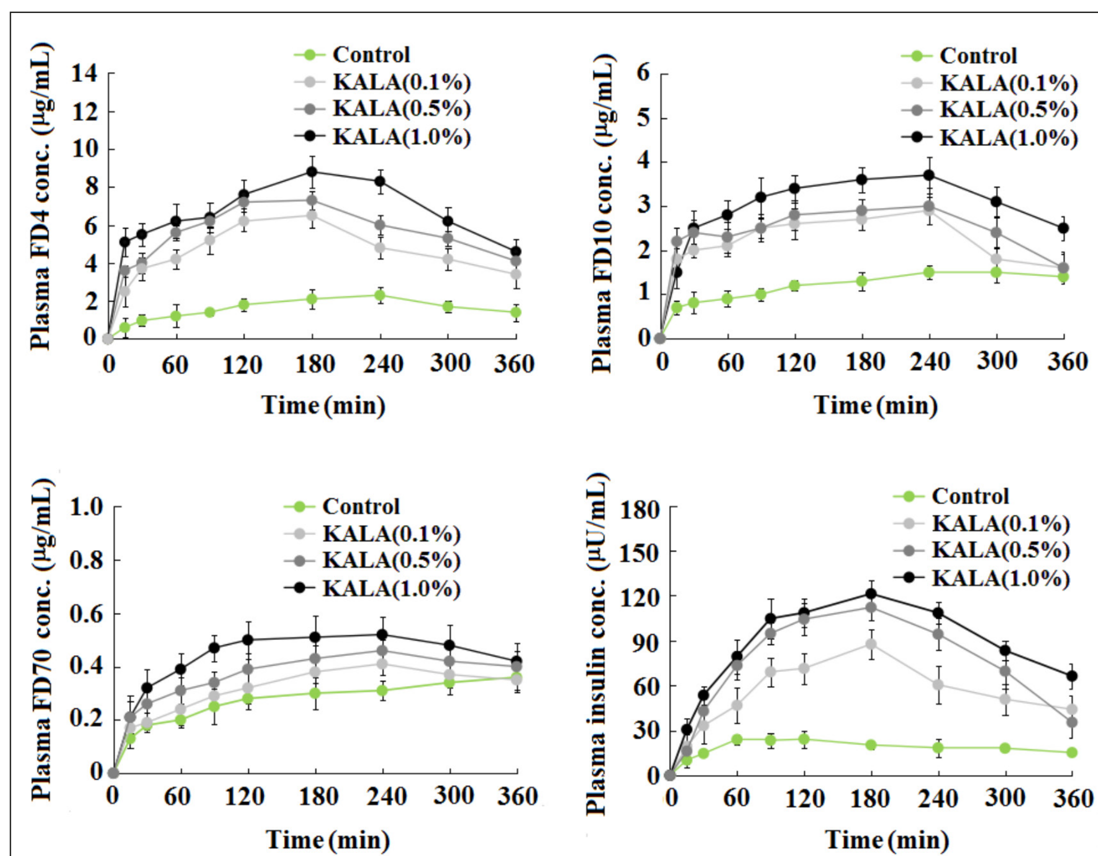

Figure 1. Concentration-time curves of FD4, FD10, FD70, and insulin in plasma after pulmonary administration to rats by means of various concentrations of lysine-alanine-leucine-alanine (KALA) peptide. Every point means mean ± S.E. of 3-5 assays.

Table II. Effect of various KALA peptide concentrations upon the absorption of FD4, FD10, FD70 and insulin from lung of rats.

	$AUC_{0\text{-}360}$ (µg min/ml)	Enhancement ratio
PBS	-	-
FD4	504.6 ± 62.7	1.0
FD4+1.0% (w/v) KALA	2,132.2 ± 327.5 **	4.2 **
FD4+0.5% (w/v) KALA	1,779.3 ± 133.4 **	3.5 **
FD4+0.1% (w/v) KALA	1,485.7 ± 144.1 **	2.9 **
PBS	-	-
FD10	352.7 ± 58	1.0
FD10+1.0% (w/v) KALA	942.8 ± 86.2 *	2.7 *
FD10+0.5% (w/v) KALA	783.6 ± 92.4 *	2.2 *
FD10+0.1% (w/v) KALA	717.3 ± 96.9 *	2.0 *
PBS	-	-
FD70	78.9 ± 18	1.0
FD70+1.0% (w/v) KALA	134.8 ± 20.4 *	1.7 *
FD70+0.5% (w/v) KALA	112.1 ± 31.9 n.s.	1.4
FD70+0.1% (w/v) KALA	95.6 ± 28.6 n.s.	1.2
	$AUC_{0\text{-}360}$ (µU min/ml)	Enhancement ratio
PBS	-	-
Insulin	6,856.5 ± 312.4	1.0
Insulin+1.0% (w/v) KALA	32,855.3 ± 2,044.1 **	4.8 **
Insulin+0.5% (w/v) KALA	28,574.6 ± 2,131.9 **	4.2 **
Insulin+0.1% (w/v) KALA	20,987.6 ± 1,865.8 **	3.1 **

The area under the curve ($AUC_{0\text{-}360}$). Data represent the mean ± S.E. of 3-5 rats. ** $p < 0.01$, * $p < 0.05$, in comparison to the control. Lysine-alanine-leucine-alanine (KALA), phosphate buffer solution (PBS), fluorescein isothiocyanate-labeled dextrans (FDs).

hind administrating with different KALA peptide concentrations. Table III demonstrated that AEEs of KALA peptide on the absorption of insulin or calcitonin by pulmonary mucosa increased with increasing KALA peptide concentrations, absorption enhancement rates of KALA peptide at concentrations of 1.0% (w/v) were 4.1 to insulin, 3.6 to calcitonin, respectively.

Effect of KALA Peptide upon the Membrane Damage to Lungs

Pulmonary membrane damage resulting from the KALA peptide was assessed by measuring the quantity of whole protein and LDH activity in BALF. As for our research, 1% (w/v) sodium deoxycholate was adopted as a positive control. As depicted in Figure 3, 1% (w/v) KALA peptide

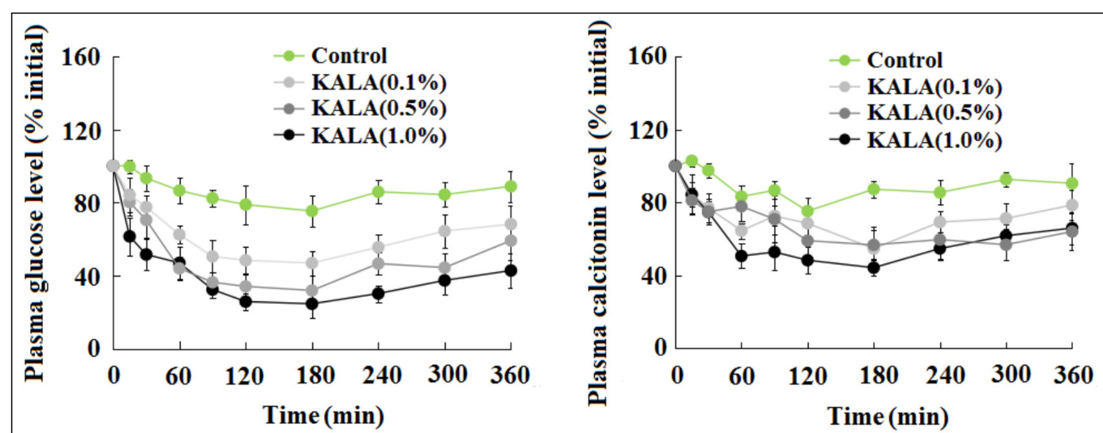

Figure 2. Concentrations-time curves of plasma glucose and calcitonin after administrating insulin and calcitonin to rats with or without varying lysine-alanine-leucine-alanine (KALA) peptide concentrations. Every point stands for mean ± S.E. of 3-5 trials.

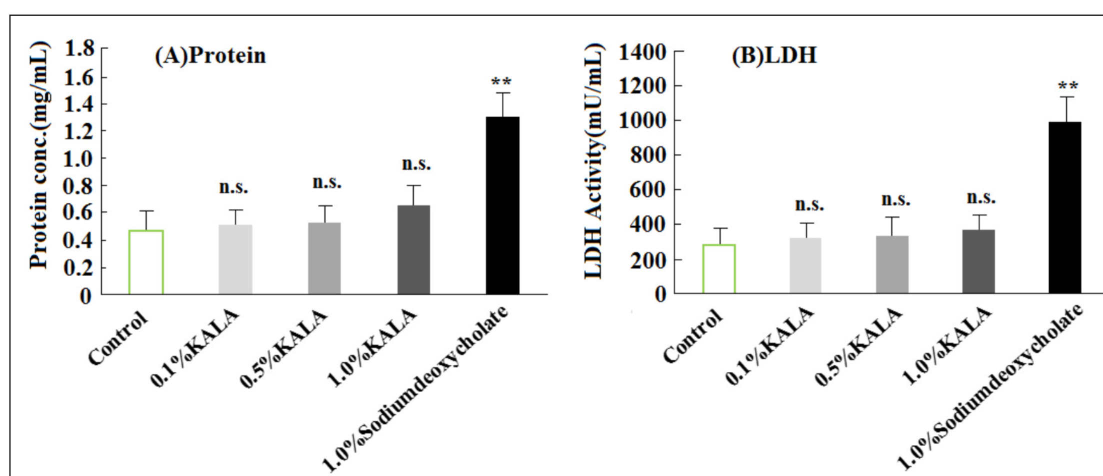

Figure 3. **A**, The quantity of protein and (**B**) lactate dehydrogenase (LDH) activity in bronchoalveolar lavage fluid (BALF) at 6 hours after intratracheal administration of lysine-alanine-leucine-alanine (KALA) peptide at different concentrations to rats. Every point stands for mean ± S.E. of 3-5 trials. ***p* < 0.01, in comparison to the PBS team as the control.

(at largest dose of administration) did not increase the quantity of protein and LDH activity in BALF, though sodium deoxycholate evidently improve the quantity of protein and LDH in BALF.

Absorption-Enhancing Mechanism of KALA Peptide

To investigate how the KALA peptide enhances the absorption of poorly absorbable medicines in the lungs, we assessed the TEER values of Xenopus pulmonary membranes at various concentrations of KALA peptide and measured KALA peptide permeability. As depicted in Figure 4, the TEER value exhibited a significant decrease with the KALA peptide compared to the control, showing a similar effect to the positive control [Ethylene Diamine Tetraacetic Acid, (EDTA)]. Also, there is no evident difference in the TEER values between different groups of KALA peptide. As shown in Figure 5, a significant increase in the p_{app} of CF was found with the KALA peptide compared to the control, and the p_{app} value was dependent on the concentration of KALA peptide in the test range from 0.1% to 1.0% w/v.

Discussion

KALA was a basic amphipathic peptide that could self-assemble into positively charged micelles[20]. In our research, it was discovered that KALA peptide at lower concentrations, from 0.1% to 1.0% w/v, enhanced the absorption of FD4, FD10, FD70, insulin, and calcitonin by pulmo-

Table III. Pharmacodynamic parameters of insulin and calcitonin after administration with different concentrations of KALA peptide from the lung of rats.

	D%	PA%	Enhancement ratio
PBS	-	-	-
Insulin	15.8 ± 2.1	9.9 ± 1.2	1.0
Insulin + 0.1% (w/v) KALA	42.3 ± 1.9	26.4 ± 0.9 **	2.7 **
Insulin + 0.5% (w/v) KALA	55.4 ± 2.4	34.6 ± 0.7 **	3.5 **
Insulin + 1.0% (w/v) KALA	64.5 ± 2.6	40.4 ± 1.6 **	4.1 **
PBS	-	-	-
Calcitonin	12.2 ± 1.6	9.4 ± 0.8	1.0
Calcitonin + 0.1% (w/v) KALA	31.9 ± 2.1	24.5 ± 1.2 **	2.6 **
Calcitonin + 0.5% (w/v) KALA	37.2 ± 1.5	28.6 ± 1.4 **	3.0 **
Calcitonin + 1.0% (w/v) KALA	44.2 ± 2.0	34.1 ± 2.2 **	3.6 **

The decrement of plasma glucose and calcium level (D%), the pharmacological availability (PA%). Data represent the mean±S.E. of 3-5 rats. ***p* < 0.01, **p* < 0.05, in comparison to the control. Lysine-alanine-leucine-alanine (KALA), phosphate buffer solution (PBS).

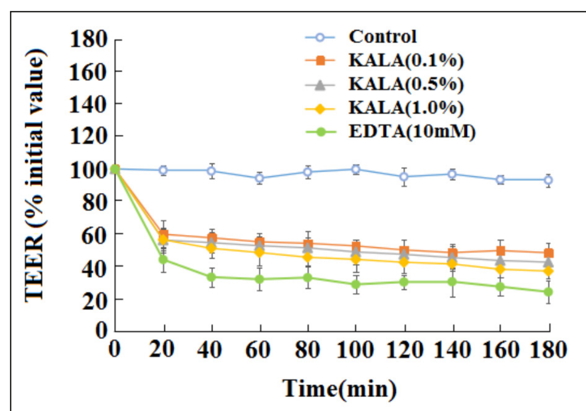

Figure 4. Effect of different concentrations of lysine-alanine-leucine-alanine (KALA) peptide on the transmembrane electrical resistance (TEER) values of the lung mucosa.

nary membranes. The AEE of the KALA peptide was concentration-dependent, and a maximum AEE was observed during the highest concentration treatment at 1 % w/v. This was likely due to the transmembrane ability and cationic properties of the KALA peptide. One possible mechanism is just that KALA peptide may make medicines traverse alveolar epithelium by means of one endocytosis pathway[30], thus enhancing the medicine absorption. Another possible mechanism is that the KALA peptide may lose a tight junction in the epithelium, thus improving the medicine delivery with one paracellular pathway[31]. Figure 6 showed a correlation line regarding molecular weight and enhancement ratio to FDs and on insulin, which indicated the absorption-enhancing ratio of KALA peptide in the lung decreased as the molecular weights of FDs increased. Nevertheless, the absorption-enhancing ratio of insulin was higher than that of FD4, although its molecular weight was greater than that of FD4. That was because, under the action of PBS (pH 7.4), insulin molecules underwent depolymerization from hexamers to dimers or monomers[32], increasing their mucosal absorption. In addition, it can be seen from Table III that although the molecular weight of insulin is greater than that of calcitonin, the absorption promotion rate of insulin is higher than that of calcitonin. Maybe it was due to the different aggregation degrees of two types of peptides in PBS (pH 7.4).

Mucosal toxicity is a key index to estimate the safety of absorption enhancers. Pulmonary mucosal toxicity caused by the KALA peptide was estimated by monitoring the changes in protein and LDH activity in BALF. Figure 3 showed that there was no obvious rise in the quantity of protein and LDH by various concentrations of KALA peptide (from 0.1 to 1% w/v). These consequences showed KALA peptide below a concentration of 1.0% w/v caused no significant membrane damage to lung tissues, and the carrier was quite safe after intrapulmonary administration. The discoveries suggested KALA peptide at lower concentrations was a safe absorption enhancer. To verify the mechanism of absorption-enhancing for KALA peptide, the data of TEER and p_{app} was detected. In TEER determination, a decrease in TEER value by the addition of KALA peptide was observed in Figure 4; the results indicated that KALA peptide decreased the TEER values by opening a tight junction in the epithelium. It could be attributed to the positive charges of KALA peptide micelles. In permeability determination, Figure 5 showed an increase in the p_{app} value of CF with

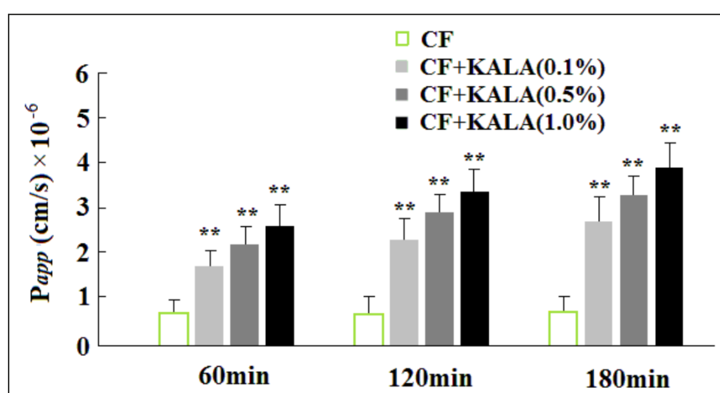

Figure 5. Effect of different concentrations of lysine-alanine-leucine-alanine (KALA) peptide on the permeation of 5(6)-Carboxyfluorescein (CF) *via* the lung mucosa. $**p < 0.01$, in comparison to the control.

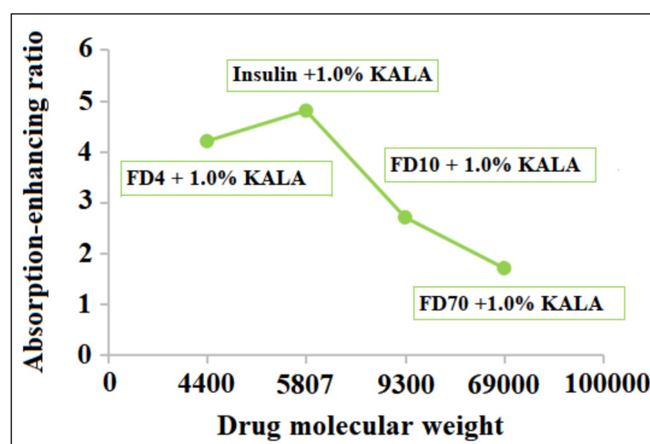

Figure 6. The relationship between absorption-enhancing rate and molecular weight of these poorly absorbable medicine solutions with 1 % (w/v) lysine-alanine-leucine-alanine (KALA) peptide. Data represent mean± S.E. of 3-5 rats.

the addition of the KALA peptide. The results suggested that KALA peptide could enhance the permeability of CF by the mucosa of the lung, maybe due to the fusogenic capability of KALA peptide. The discoveries further confirmed KALA peptide could enhance pulmonary absorption of poorly absorbable medicines through the pathways of paracellular and transcellular[33,34].

Conclusions

According to our findings, the KALA peptide, when present in concentrations ranging from 0.1% to 1.0%, enhanced the absorption of insulin and calcitonin by pulmonary membranes. This led to decreased levels of plasma insulin and calcium compared to the control group. The effect of the KALA peptide on absorption efficiency was found to be dependent on its concentration. The test value of protein and LDH in BALF did not increase with KALA peptide with various concentrations, showing that KALA peptide caused no membrane damage to the lungs. A decrease in TEER value and an increase in p_{app} value by the addition of KALA peptide indicated that KALA peptide had the ability to aid drug delivery through epithelial cells *via* two routes of paracellular and transcellular. In conclusion, KALA peptides are suitable as an absorption enhancer below a concentration of 1.0% w/v for improving the pulmonary absorption of numerous poorly absorbable medicines, particularly peptide and protein medicines, without any measurable cytotoxicity.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

C.Y. Yan: conceptualization, methodology, project administration, writing-original draft. Z.L. Chen and M.Y. Wan: data curation, validation, formal analysis, investigation. J.W. Gu: software. All authors approved the final manuscript.

Ethics Approval

All animal experiments were approved by the Animal Ethics Committee at Guili Medical University (GLMC202103195, 09-03-2021).

Informed Consent

Not applicable.

Availability of Data and Materials

The combined datasets and materials were available upon reasonable request.

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AI Disclosure

This study, including its figures, was conducted without the use of artificial intelligence or any assisted technologies.

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