Rubiscolin-6 rapidly suppresses the postprandial motility of the gastric antrum and subsequently increases food intake via δ-opioid receptors in mice

KOJI ATAKA[1], AKIHIRO ASAKAWA[2] and IKUO KATO[1]

[1]Laboratory of Medical Biochemistry, Kobe Pharmaceutical University, Kobe 658-8558;
[2]Department of Psychosomatic Internal Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544, Japan

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Abstract. Rubiscolin-6 is a food-derived opioid peptide found in *Spinacia oleracea* that has anti-nociceptive, memory-enhancing, anxiolytic-like and anti-depressant effects. Rubiscolin-6 has been reported to have two opposing effects on food intake. Food intake is closely connected to gut motility; however, to the best of our knowledge, the effect of rubiscolin-6 on gut motility has not been reported. The present study aimed to investigate the effect of rubiscolin-6 on postprandial motility of the gastric antrum in conscious mice. A catheter was implanted in the gastric antrum of male C57BL/6J mice. Manometric measurements were performed in fasted male mice and chow was then provided to assess motility in the fed state. Rubiscolin-6, the δ-opioid receptor antagonist naltrindole, a mixture of rubiscolin-6 and naltrindole, or vehicle was administered intraperitoneally 30 min after eating. The percentage motor index (%MI) was then calculated. Cumulative food intake was measured in both *ad libitum*-fed and overnight-fasted mice. The %MI was significantly lower in mice treated with rubiscolin-6 compared with that in the other groups, but normalized by treatment with the rubiscolin-6/naltrindole mixture. The decrease in %MI induced by rubiscolin-6 remained for 1 h after administration. Cumulative food intake was significantly higher 4 and 6 h after rubiscolin-6 administration in *ad libitum*-fed mice but was normalized by the rubiscolin-6/naltrindole mixture. Food intake 30 min after rubiscolin-6 administration was normal, but was higher in mice treated with the rubiscolin-6/naltrindole mixture. Thus, rubiscolin-6 may have a rapid effect to reduce postprandial antral motility and may subsequently increase food intake after this inhibitory effect disappears. These effects were revealed to be mediated through δ-opioid receptors. The orexigenic effect of rubiscolin-6 may be applicable to the treatment of anorexia and cachexia.

Correspondence to: Dr Koji Ataka, Laboratory of Medical Biochemistry, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan
E-mail: kataka@kobepharma-u.ac.jp

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Introduction

Various food-derived peptides have been shown to be beneficial for human health (1). Food-derived opioid peptides, which are categorized as exogenous bioactive peptides, have been expected to have beneficial effects on psychosomatic disorders, such as depression, anxiety, pain, eating disorders, and stress-related disorders, and to have no notable side effects (2). In general, exogenous food-derived opioid peptides are obtained by the enzymatic degradation of food in the intestines, and are therefore resistant to breakdown by intestinal enzymes (2).

Rubiscolin-6 is derived from D-ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco), a key enzyme in the Calvin–Benson carbon fixation cycle in plants. Rubiscolin-6 is composed of six amino acids: Tyr-Pro-Leu-Asp-Leu-Phe (3). The total mass of the rubisco enzymes is ~0.7 Gt in the terrestrial environment (~3% of total leaf mass) and ~0.03 Gt in the marine environment (4), and accounts for approximately 30-50% of the soluble protein present in the green leaves of plants (5). Rubiscolin-6 has been shown to have several beneficial effects, including anti-nociceptive, memory-enhancing, anxiolytic-like, and anti-depressant effects (3,6-8). It has also been shown to increase the consumption of a normal diet, but decrease that of a high-fat diet (9,10). Rubiscolin-6 is a δ-opioid peptide, and these activities are thought to be mediated via δ-opioid receptor agonism. Furthermore, rubiscolin-6 has been shown to cross the blood-brain barrier (2-4). It well known that δ-opioid receptor activation contributes to food intake and gastrointestinal function (11-15). However, the effect of rubiscolin-6 on intestinal motility has not been determined.

Gastric antral motility is closely connected with food intake, and appetite regulation involves a balance between hunger and satiety. The stomach responds to the mechanical stimuli caused by meal volume and composition and transmits information to the hypothalamus, which is an important area of

brain to control food intake and gastrointestinal function, via the vagal nerve and gut hormone secretion (16). Furthermore, gastric motility, such as fundic compliance (gastric accommodation) and antral contractions (gastric emptying) contribute to the regulation of the balance between hunger and satiety (17). The distal stomach, including the antrum, influences satiety, which is the feeling to cause the termination of eating (18). Acyl ghrelin, a peptide produced by the stomach, induces a fasting motor pattern of antral motility in the fed state (19,20), whereas des-acyl ghrelin, the precursor of acyl ghrelin, suppresses a fasting motor pattern of antral motility in the fasted state (21). In addition, acyl ghrelin stimulates, and des-acyl ghrelin suppresses food intake (21,22).

In the present study, we aimed to investigate the effects of rubiscolin-6 on the postprandial antral motility of conscious mice using a manometric method and on the food intake of *ad libitum*-fed and overnight-fasted mice. In addition, we assessed the effects of a δ-opioid receptor antagonist on the rubiscolin-6-induced changes in antral motility and food intake.

Materials and methods

Rubiscolin-6 synthesis and purification. Rubiscolin-6 (YPLDLF) was synthesized by a solid-phase fluorenylmethyloxycarbonyl (Fmoc)-based strategy and an automated peptide synthesizer (Model Pioneer; Thermo Fisher Scientific), as previously described (23). The crude peptide was purified by reverse-phase high performance liquid chromatography (HPLC, Delta 600 HPLC System) using a Develosil ODSHG-5 column (2x25 cm; Nomura Chemical Co., Ltd.). High purity of the purified peptide was confirmed by analytical HPLC and MALDI-TOF MS analysis.

Animals. Seven-week-old male C57BL/6J mice were purchased from Japan SLC, Inc. The mice were individually maintained in a pathogen-free facility at 24±2˚C and 50±10% humidity, under a 12-h/12-h light/dark cycle (lights on 07:00 a.m. to 07:00 p.m.), with *ad libitum* access to sterile standard chow (3.4 kcal/g; CE-2, CLEA Japan Inc.) and water, in the animal facility of Kobe Pharmaceutical University. All the animal protocols were approved by the Kobe Pharmaceutical University Committee for Animal Experiments (approval no. 2021-063) and performed in accordance with the relevant guidelines and regulations.

Catheter implantation for manometric recording. Catheter implantation was performed as previously reported (24). The mice were anesthetized by the intraperitoneal (ip) administration of a mixture of 0.3 mg/kg of medetomidine (Domitor, Meiji Seika Pharma), 4.0 mg/kg of midazolam (Sandoz), and 5.0 mg/kg of butorphanol (Vetorphale, Meiji Seika Pharma). A mixed anesthetic agent has been recommended for animal experiments in Japan, replacing ketamine (25), which has been categorized as a narcotic drug by the Japanese Narcotics Control Law and is no longer easy to access for use in animal experiments in Japan. This mixed anesthetic agent has been used in servals (26), cats (27), and dogs (28) outside of Japan. A silicone tube (ID 0.3, OD 0.6 mm; Access Technologies) was inserted into the stomach through a small incision in the gastric body, and the tip was placed in the gastric antrum. The tube was fixed to the gastric wall using a purse-string suture, run subcutaneously to emerge in the dorsal neck, and secured to the skin. After implantation, ip administration of 0.3 mg/kg of atipamezole (Antisedan; Nippon Zenyaku Kogyo) was used to reverse the anesthesia and the mice were allowed to recover in individual cages for 7 days.

Measurement of antral motility and experimental protocols. The mice were deprived of food for 18 h before the experiment. On the day of the experiment, they were placed individually in a black box (150x200x300 mm) with an open top. The manometric catheter placed in the stomach was connected to an infusion swivel (375/D/20, Instech Laboratories) on a single-axis counter-weighted swivel mount (TSB-23, Eicom) to allow free movement, and then joined to a pressure transducer (DX-100, Nihon Koden Kogyo). The catheter was then continuously infused with bubble-free distilled water at 25 μl/h using an infusion pump (NE-1600, New ERA Pump System). Data were recorded and stored in a PowerLab (AD Instruments). The basal motor patterns in the antrum were monitored during the experiments. The mice were given 0.3 g of laboratory chow, which they all consumed within 30 min. Thirty minutes after the mice finished eating the pellet, they were intraperitoneally administered 0.1 ml phosphate-buffered saline (PBS) pH 7.0 (vehicle), 0.1 or 0.3 mg/kg rubiscolin-6 dissolved in PBS, 1 mg/kg naltrindole hydrochloride (a δ-opioid receptor antagonist, 111469-81-9; Tocris Bioscience) in PBS, or a mixture of 0.3 mg/kg rubiscolin-6 and 1 mg/kg naltrindole in PBS. The percentage motor index (%MI) of the fed motor activity in the antrum was calculated as (area under the manometric trace for each 20-min period after treatment)/(area under the manometric trace for the 20-min period immediately before treatment) x 100. Changes in the mean value of %MI for each 20-min period between 0-20, 20-40, 40-60, 60-80, and 80-100 min after the ip administration of 0.3 mg/kg of rubiscolin-6 or 0.1 ml of vehicle were compared. We performed catheter implantation in 33 mice to measure the antral motility, which were maintained without abnormal behavior (e.g., immobility, tremors) and euthanized with the inhalation anesthesia of 6-8% isoflurane (FUJIFUILM Wako Pure Chemical Co.) after the experiments. The inhalation of anesthesia was maintained until euthanasia was confirmed by respiratory and cardiac arrest.

Measurement of food intake and experimental protocols. Measurements of food intake commenced at 10:00 a.m. in both *ad libitum*-fed and overnight-fasted mice. Cumulative food intake was measured 1, 2, 4, and 6 h after the ip administration of 0.1 ml vehicle, 0.3 mg/kg rubiscolin-6, 1 mg/kg naltrindole, or a mixture of 0.3 mg/kg rubiscolin-6 and 1 mg/kg naltrindole. Food intake after the ip administration was also measured for 30 min in other mice.

Statistical analysis. Data are expressed as mean ± SEM and were analyzed using Prism software (version 9.3.1.; GraphPad). Multiple groups were compared using one- or two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Differences were considered to be statistically significant when $P<0.05$.

Results

Effects of rubiscolin-6 on postprandial motility in antrum. The ip administration of 0.3 mg/kg rubiscolin-6 caused a change in antral motility (Fig. 1Aa, b): the %MI in mice treated with 0.3 mg/kg rubiscolin-6 was significantly lower than that of mice treated with vehicle (Fig. 1C). However, no difference in %MI was identified in mice treated with 0.1 mg/kg of rubiscolin-6. There were no differences in the traces of mice treated with 1 mg/kg naltrindole, a δ-opioid antagonist, or the mixture of 0.3 mg/kg rubiscolin-6 and 1 mg/kg naltrindole (Fig. 1Ba, b): the %MI of mice treated with the mixture of 0.3 mg/kg rubiscolin and 1 mg/kg naltrindole was similar to that of vehicle-treated mice (Fig. 1C).

Effect of rubiscolin-6 on food intake. The cumulative food intake of *ad libitum*-fed mice treated with 0.3 mg/kg rubiscolin-6 was significantly higher 4 and 6 h after administration than in the other groups (Fig. 2A). The cumulative food intake of mice treated with the mixture of 0.3 mg/kg rubiscolin-6 and 1 mg/kg naltrindole was significantly lower than that of mice treated with 0.3 mg/kg rubiscolin-6 at these time points (Fig. 2A). However, the food intake for 30 min of mice treated with 0.3 mg/kg rubiscolin-6 did not differ from that of mice treated with vehicle (Fig. 2B). In addition, the food intake over 30 min of mice treated with the mixture of 0.3 mg/kg rubiscoln-6 and 1 mg/kg naltrindole was significantly higher than that of the other groups (Fig. 2B). There were no significant differences among the groups of fasted mice (Fig. 2C).

Duration of the inhibitory effect of rubiscolin-6. Because food intake was high in mice treated with 0.3 mg/kg rubiscolin-6 4 and 6 h after administration, and the food intake for 30 min after the administration of the mixture of 0.3 mg/kg rubiscolin-6 and 1 mg/kg naltrindole was also high, we measured %MI over time. We found that %MI was significantly reduced for each 20-min period between 0-20, 20-40, 40-60 min after the ip administration of rubiscolin-6 (Fig. 3).

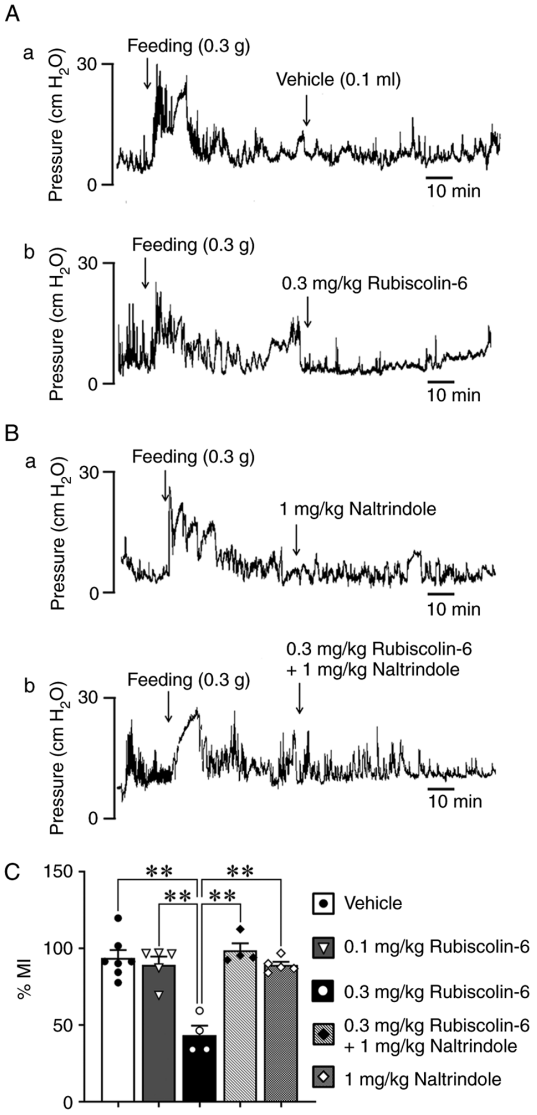

Figure 1. Effects of rubiscolin-6 and a mixture of rubiscolin-6 and a δ-opioid receptor antagonist, naltrindole, on the antral motor activity in the postprandial state of mice. Representative antral motility traces from mice that underwent intraperitoneal (ip) administration of vehicle (Aa), 0.3 mg/kg rubiscolin-6 (Ab), 1 mg/kg naltrindole (Ba), or a mixture of 0.3 mg/kg rubscolin-6 and 1 mg/kg naltrindole (Bb) are shown. (C) Effects of the ip administration of rubiscolin-6 and a mixture of rubiscolin-6 and naltrindole on the change in the percentage motor index (%MI) over the subsequent 20 min. Values are mean ± SEM (n=4-7). **P<0.01. %MI, percentage motor index.

Discussion

Endogenous opioids, such as methionine enkephalin, leucine enkephalin, β-endorphin, and dynorphin, are expressed in enteric neurons and mucosal endocrine cells, and these have effects through μ-, κ-, and δ-opioid receptors (29). Opioid-induced bowel dysfunction is a well-known adverse effect of opioid use, and opioid-induced constipation is the most common form of this (15). Many previous studies have investigated the effect of opioids, especially μ-opioid agonists, on small intestinal and colonic function, but there have been few studies of the effects of δ-opioids on stomach motility. Holle and Steinbach demonstrated that a δ-opioid receptor antagonist, ICI 174 864, increases antral motility in the postprandial state in conscious dogs (30). In addition, Ruckebusch *et al* demonstrated that D-AIa^2-MetS-enkephalinamide (DAMA) and D-Ala^2-D-LeuS-enkephalin (DADLE), which are mixed μ and δ-opioid agonists, inhibit the motility of the reticulum in sheep (13). We have shown that rubiscolin-6 suppresses the postprandial motility of the antrum in mice, and that this inhibitory effect is reduced by a δ-opioid receptor antagonist. Thus, rubiscolin-6 inhibits postprandial antral motility, and this effect may be mediated by the δ-opioid receptor.

Porreca *et al* found that the δ-opioid receptor agonists [D-Pen^2, L-Pen^5] enkephalin (DPLPE) and [D-Pen^2, D-Pen^5] enkephalin (DPDPE) inhibit gastrointestinal ^{51}Cr transit following intrathecal administration but not intracerebroventricular (icv) administration in mice (31). In addition, Galligan *et al* demonstrated that icv administration of DPLPE or DPDPE does not inhibit gastrointestinal ^{51}Cr transit in mice (32). However, Ruckebusch *et al* demonstrated that the effects of both μ and δ-opioid opioid agonists on reticular motility are prevented by the administration of naloxone, which has high affinity for μ-opioid receptors, but not by

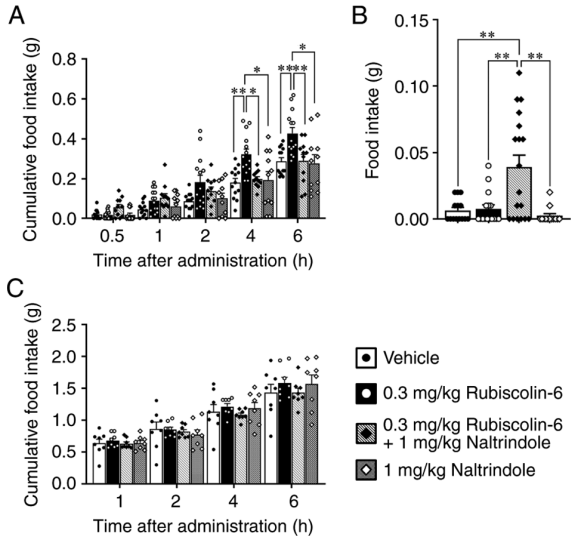

Figure 2. Effects of rubiscolin-6 and a mixture of rubiscolin-6 and a δ-opioid receptor antagonist, naltrindole, on the food intake of mice. (A) Cumulative food intake of *ad libitum*-fed mice that underwent intraperitoneal (ip) administration of vehicle, 0.3 mg/kg rubiscolin-6, 1 mg/kg naltrindole, or a mixture of 0.3 mg/kg rubscolin-6 and 1 mg/kg naltrindole (n=11-13). (B) Food intake over 30 min of *ad libitum*-fed mice that underwent ip administration of vehicle, 0.3 mg/kg rubiscolin-6, 1 mg/kg naltrindole, or a mixture of 0.3 mg/kg rubscolin-6 and 1 mg/kg naltrindole (n=13-18). (C) Cumulative food intake of fasted mice that underwent ip administration of vehicle, 0.3 mg/kg rubiscolin-6, 1 mg/kg naltrindole, or a mixture of 0.3 mg/kg rubscolin-6 and 1 mg/kg naltrindole (n=8). Values are mean ± SEM. *P<0.05, **P<0.01.

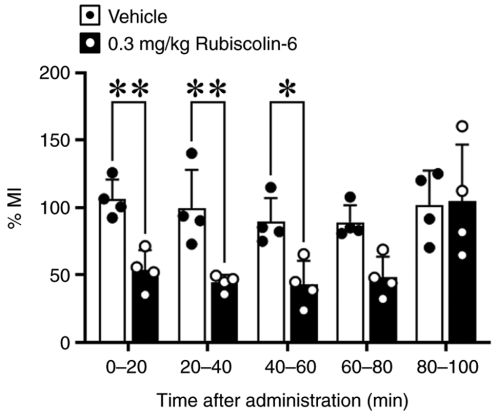

Figure 3. Changes in the percentage motor index (%MI) in the antrum in the periods 0-20, 20-40, 40-60, 60-80 and 80-100 min after administration of vehicle or rubiscolin-6. Values are the means ± SEM (n=4). *P<0.05, **P<0.01. %MI, percentage motor index.

the quaternary parent compound methylnaloxone, which is a derivative of the opioid receptor antagonist naloxone and cannot cross the blood–brain barrier (13). Poole *et al* demonstrated that δ-opioid receptors localized in the stomach using knock-in mice carrying a green fluorescent protein linked to Oprd1, which encodes the δ-opioid receptor (33). In addition, Wittert *et al* reported that δ- and κ-opioid receptors, not μ-opioid receptors, were detected in the stomach, and all three opioid receptor subtypes were detected in the hypothalamus of rodents using polymerase chain reaction analysis (34). These results indicate that δ-opioid receptors in the stomach

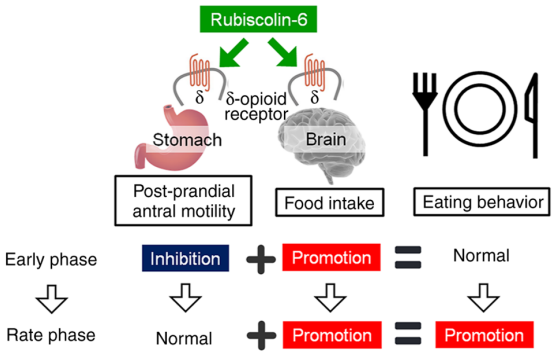

Figure 4. Schematic diagram of the relationship between the inhibitory effect of rubiscolin-6 on antral motility and its positive effect on food intake.

and μ-receptors in the brain influence stomach motility. Similarly, the present findings imply that the inhibitory effect of rubiscolin-6 on antral motility may be mediated by δ-opioid receptors in the stomach.

Food intake is regulated by the hypothalamus, where various neurons that secrete orexigenic and anorexigenic peptides are located. The hypothalamus receives various stimuli from peripheral tissues via the vagal nerves and hormones secreted into the blood circulation; e.g., leptin from the adipose tissue, insulin from the pancreas, and acyl ghrelin from the stomach (35). Gosnell *et al* demonstrated that the icv administration of a selective δ-opioid receptor agonist, [D-Ser^{2}, Leu^{5}, Thr^{6}]-enkephalin (DSLET), increases food intake in *ad libitum*-fed rats (11). Israel *et al* indicated that δ-, κ-, and μ-opioid receptors contribute to the neuropeptide Y (NPY)-induced food intake (36). This potent orexigenic neuropeptide in the hypothalamus regulates feeding behavior and energy homeostasis (37). Furthermore, Kaneko *et al* proposed that the effect of rubiscolin-6 to increase food intake is mediated by the activation of NPY neurons (10); i.e., the effect of rubiscolin-6 on food intake might be mediated centrally. However, food intake is also regulated by gastrointestinal motility (16). Des-acyl ghrelin reduces both antral motility and food intake (21). In the present study, rubiscolin-6 was shown to significantly reduce postprandial antral motility for 1 h, whereas the orexigenic effect of rubiscolin-6 started to appear after 2 h, and was significant 4 and 6 h after administration. In addition, although the administration of rubiscolin-6 or a δ-opioid receptor antagonist alone had no effect, a mixture of rubiscolin-6 and δ-opioid receptor antagonist significantly stimulated food intake for 30 min. These findings suggest that the inhibitory effect of rubiscolin-6 on antral motility may predominate over the orexigenic effect and may contribute to the rapid loss of the effect on food intake. Thus, the increase in food consumption may appear after the inhibitory effect on the antral motility disappears (Fig. 4). However, there are a few limitations of the present study. It is not clear whether the central effects of rubiscolin-6 are mediated by a direct, neuronal, or hormonal pathway. The relationship between the inhibitory effect of rubiscolin-6 on antral motility and its effect on food intake, and the roles of central and peripheral δ-opioid receptors in these effects, require further study. The inhibitory effect of rubiscolin-6 on antral motility may cause stomach discomfort and upset. The effects of rubiscolin-6 need to be

evaluated with healthy participants and patients with anorexia and cachexia.

We have shown that rubiscolin-6 inhibits postprandial antral motility for the first time and that it promotes food intake in the fed state through δ-opioid receptors. Furthermore, the inhibitory effect of rubiscolin-6 on postprandial antral motility may delay the appearance of its effect on food intake. The orexigenic effect of rubiscolin-6 may be applicable to the treatment of anorexia and cachexia, which are characterized by severe reductions in food intake and appetite.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

IK and AA designed the study. IK synthesized the peptide. KA carried out all experiments, collected the data, analyzed the data and wrote the manuscript. IK reviewed the manuscript. IK, AA and KA confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal protocols for this study were approved by the Kobe Pharmaceutical University Committee for Animal Experiments (2021-063). Experiments were performed in accordance with the relevant guidelines and regulations.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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