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Chitosan dosage regimen to trap fecal oil excretion after peroral lipase inhibitor administration in mice

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ABSTRACT

This study was designed to investigate the oil entrapment and systemic oil absorption-reducing activities of chitosan. High-molecular-weight chitosan formed gel aggregates with oil and bile salts *in vitro*. The oil/chitosan ratio and the molecular weight of chitosan were optimized for the *in vivo* study, and a molecular weight >100,000 was effective in reducing the oil contamination of mouse fur. The oil/chitosan weight ratio required for effective oil entrapment was less than 13 and 5 in the *in vitro* and *in vivo* experiments, respectively. Chitosan administration was most effective during meals, and high-molecular-weight chitosan could trap and facilitate the reduction of systemic absorption of oil droplets separated by orlistat. The activity of the lipase inhibitor was not altered by chitosan as evidenced by thin layer chromatography, and orlistat was not absorbed systemically by the co-administration of chitosan.

1. Introduction

Chitosan is a biopolymer widely used as a dietary supplement, with a fat binding activity attributable to its positive charge [1]. Oils possess negative charge owing to the presence of fatty acids; therefore, they interact with chitosan by electrostatic attraction. Since chitosan can bind to fat [2,3], numerous research studies on the prevention of dietary fat absorption by chitosan, and the feasibility of its utilization for the treatment of obesity have been performed [4–7]. However, previous studies have reported conflicting outcomes [8–10]. It has been reported that chitosan improved humoral and cellular immune responses as well as weight gain in weaned pigs by increasing the serum concentrations of growth hormone and improving the morphological structure of the small intestine [11–14]. In contrast, several animal studies have shown that chitosan reduced weight gain [4,6,15–27]. Furthermore, clinical trials have also reported that chitosan acted as a dietary fiber, and it was very effective in treating overweight or obese subjects by reducing the body weight gain and absorption of excess body fat [7,28–30]. However, other clinical trials demonstrated opposing results, showing that chitosan had no or little effect on body weight, with no statistically significant difference in obesity control and no therapeutic reduction of body weight [31–35]. In particular, a series of studies by Gades et al. have shown that the fat trapping ability of chitosan in men and women was unsubstantiated because it did not increase fecal fat excretion [32,33,35].

In most *in vivo* studies investigating the fat-binding activity of chitosan, a standard or high-fat diet containing chitosan was fed to the experimental animals *ad libitum* [5,15–17,19–23,25–27]. Based on these experimental conditions, the amount of fat and chitosan consumed by each experimental animal was not strictly controlled, and it was difficult to control the gastrointestinal tract environment where the chitosan and oil emulsification occurred. In a previous animal experiment, butter emulsion and/or chitosan solution were orally administered to the mice to control the dose accurately, and chitosan was effective in reducing the plasma triacylglycerol concentrations and body weight of mice [6,18,24].

In this study, the doses of chitosan, oil, and orlistat, as well as the molecular weight of chitosan and the dosing sequence and time, were investigated systematically. This was performed to establish the exact dosing regimen that can effectively reduce fecal contamination without reducing the oil excretion in experimental animals. Orlistat is a weight-loss agent, which inhibits gastric and pancreatic lipases in the gastrointestinal tract to reduce the systemic absorption of fat [36]. High-molecular-weight chitosan fed to the animals along with oil and orlistat localized the oil inside the feces without contaminating the fur and cages when the proper feeding sequence and timing were followed.

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2. Materials and methods

2.1. Materials

Chitosan was purchased from Wako Pure Chemical Industries (Osaka, Japan) and kindly provided by Biopolytech (Cheongwon, Korea). The molecular weights of Wako water-soluble chitosan, Wako 50 chitosan, Biopolytech chitosan 9, Biopolytech chitosan 17, and Wako 100 chitosan were 3.4, 50, 100, 500, and 750 kDa, respectively. Orlistat used in this study was a product of Biocon (Bangalore, India) while acetic acid and *N*-methyl-2-pyrrolidone (NMP) were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Soybean oil, sudan IV, sodium taurodeoxycholate hydrate, potassium phosphate, dimethyl sulfoxide, chloroform, triolein, diolein, oleic acid, lipase, polysorbate 80, iodine, itraconazole, ammonium acetate, and formic acid were obtained from Sigma-Aldrich (St Louis, MO, USA). Sodium hydroxide (NaOH) was purchased from Duksan Pure Chemicals Co., LTD. (Ansan, Korea). Monoolein was purchased from Danisco Ingredients (Copenhagen, Denmark) while acetonitrile was purchased from J. T. Baker (Phillipsburg, NJ, USA). Isopropyl alcohol, diethyl ether, and hexane were obtained from Daejung Chemicals & Metals Co., Ltd. (Siheung, Korea). The soybean oil samples used in this study were prepared by dissolving a colorimetric probe, sudan IV, at 2 mg/mL. The density of soybean oil was 0.917 g/mL. All experiments in this study were performed by using this soybean oil sample containing sudan IV. The extraction solvent was prepared by mixing chloroform and isopropyl alcohol (1:19, v/v). The artificial bile salt was prepared by dissolving sodium taurodeoxycholate hydrate at 0.01 M in 0.1 M potassium phosphate at pH 6.8.

2.2. Preparation of purified chitosan powder

Wako 50 and 100 chitosan solutions were prepared by dissolving the samples in 1% aqueous acetic acid solution followed by pressure filtration to remove the insoluble residues. Excess acetic acid was removed through dialysis by using a semi-permeable cellulose dialysis membrane (molecular weight cut-off: 6–8 kDa) for a day. Then, the solution was freeze-dried to obtain the purified chitosan powder, which was readily water-soluble [2,3].

2.3. In vitro *interaction between chitosan and oils*

Chitosan solutions were prepared using 3.4, 50, 100, 500, and 750 kDa of chitosan as 1% (w/v) solutions in water. A solution containing 0.01 M sodium taurodeoxycholate hydrate at 0.01 M in potassium phosphate buffer was used as artificial bile. Chitosan solution, artificial bile, and soybean oil were mixed at a ratio of 1:1:0.02 (v/v/v) for observation. Furthermore, mixtures without artificial bile or oil were prepared by adding an equal volume of water instead. The mixtures were shaken and then sonicated for 30 s before adding water or artificial bile. The dispersions were observed in tubes with the naked eye and under a light microscope (Olympus DP70, Olympus Co., Tokyo, Japan) at ×100 magnification.

2.4. Oil-dispersing capability of chitosan at various temperature and pH conditions

Chitosan solutions were prepared using 3.4, 50, 100, 500, and 750 kDa of chitosan as 2% (w/v) solutions in water. The pH values were adjusted to 2 and 7.4 by using acetic acid and 0.1 M NaOH, respectively. The chitosan solution and soybean oil containing sudan IV (2 mg/mL) were mixed at a ratio of 1:1 (v/v) by sonicating for 2.5 min. The mixtures were then incubated at 4, 25, and 37 °C for a day. The oil phase separated from the mixture was removed to obtain homogeneous dispersion solubilizing oil. To measure the amount of oil, the dispersed oil in the chitosan solutions was extracted by vortexing with the extraction solvent, which consisted of a mixture of chloroform and isopropyl alcohol at 1:19 (v/v). For the detection and quantification of sudan IV, the absorbance was measured at 520 nm using a spectrophotometric microplate reader (SpectraMax 340, Molecular Devices, Sunnyvale, CA, USA). The amount of oil was calculated by quantifying the sudan IV content as described previously [37].

2.5. Animal experiments

Seven-week-old female BALB/c mice (18–20 g) were purchased from Orient Co., (Seongnam, Korea) and housed at five animals per cage (260 × 200 × 130 mm; Jeung Do Bio & Plant Co., Seoul, Korea). They were allowed to acclimatize for a week before the experiment under a 12-h light/dark cycle, and they were given *ad libitum* access to irradiated pelleted food (Harlan Teklad, Madison, WI, USA) and sterilized drinking water. Animal care and handling in this study followed the institutional guidelines of the Animal Care and Use Committee of the Korea Institute of Science and Technology (2014-058).

A vest-collar [38] was placed on each mouse for 1 day to familiarize it prior to the experiments. This vest-collar was vital to the experiment for quantifying the oil produced because it prevents the animal from grooming and licking the parts of the body where oil is likely to be smeared. The vest-collar was removed during oral feeding of the test materials to the mice, and then it was placed back. The mice were housed individually in mini mouse cages (143 × 256 × 127 mm, Jeung Do Bio & Plant Co.) during the measurement of fecal oil excretion. Their feces and contaminated fur were collected 15 h after the administration of the test materials, and the collected samples were completely oven-dried before analysis ($n = 5$).

2.6. Analysis of excreted oil in feces and on fur

To measure the oil excreted in the feces and on the fur, sudan IV, an oil-soluble dye, was used as a probe for the visual confirmation of the sample collected and quantitation of oil. Soybean oil was mixed with sudan IV, since it has been proven to be a good indicator for tracking oil *in vivo* [37]. The mice were fed with various test materials along with the oil containing sudan IV. The fecal samples were collected and homogenized with chloroform and isopropyl alcohol mixture (1:19, v/v) at 16.7% (w/v) using a homogenizer (T25 basic, IKA Labortechnik, Staufen, Germany), and then centrifuged at 10,000 × *g* for 5 min at 4 °C. Then, the supernatant was collected in a new tube by filtration. The oil on the fur samples was extracted by vortexing with the extraction solvent (16.7%, w/v), followed by centrifugation at 5000 × *g* for 5 min at 4 °C. The supernatant was subsequently collected by filtration. The absorbance of sudan IV was measured as mentioned above in Section 2.4.

2.7. Thin layer chromatography (TLC)

The oil standard was prepared by mixing equal weights of triolein, diolein, monoolein, and oleic acid. Chitosan (750 kDa) and orlistat solutions were prepared at 3% (w/v) in water and 0.2% (w/v) in dimethyl sulfoxide, respectively. Orlistat dispersion in chitosan (750 kDa) solution was prepared by mixing them at a ratio of 1:15 (w/v), and then adding triolein to each sample (1:4, v/v). All samples, including the oil standard, were mixed with artificial bile salt (1:1, v/v), and then incubated for 30 min at 37 °C in a shaking water bath. Each mixture was subsequently added to 0.1% lipase or water, which was then incubated for 5 min at 37 °C in a shaking water bath. Thin layer chromatography (TLC) was performed after removing water from each mixture by solvent extraction with a