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Biotechnological and microbiological aspects of development of capsule form for fecal microbiota transplantation

Alexandra Vadimovna Melikhova [1,*], Elena Nikolaevna Ilina [1], Denis Yuryevich Mokhirev [1], Yana Dmitrievna Benediktova [1], Alexander Vladimirovich Pavlenko [1], Veronika Igorevna Polyakova [2], Elena Vladimirovna Cherkashina [2], Kaytsak Vartanovich Simavonyan [2] and Ilona Murmanovna Arshba [2]

[1] *Research Institute for Systems Biology and Medicine (RISBM), Moscow, Russia. Nauchny proezd, 18, Moscow, 117246, Russia.*
[2] *Kurchatov Complex of Medical Primatology of NRC "Kurchatov Institute". Akademika Lapin Street, 177, Veseloye village, Sochi, 354376, Russia.*

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Abstract

Aim: To evaluate the feasibility of freeze-drying the substance of monkey gut microbiota and the use of serial acid-tolerant capsules for the preparation of a finished form for fecal microbiota transplantation (FMT).

Materials and Methods: To prepare a liquid substance for fecal microbiota transplantation, monkey feces were homogenised in physiological solution, then centrifuged to obtain the supernatant, which was stabilised by adding 5% sucrose. The obtained suspension was poured into vials and frozen at -80 °C. After that, the substance was dried in Labconco's FreeZone 6 freeze-drying unit, crushed and filled into capsules using a PRESSORE 100 capsule machine.

To protect the capsules from the acidic environment of the stomach, tests were carried out using barium sulphate. The capsules were examined radiologically by introducing them into the mouths of the animals and taking radiographs of the gastrointestinal tract in two projections. Molecular genetic studies were carried out according to the methodological instructions for working with nucleic acids. Intestinal suspensions of microbiota were prepared using sterile phosphate buffer. Nucleic acid extraction and PCR were performed using DNA-Sorb-B reagents and the COLONOFLOR-16 kit, with detection on a CFX-96 device.

Results: The preparation of capsules for fecal microbiota transplantation requires a dry active substance of the microbiota. The lyophilisation process was carried out at -37°C for 32 hours. DR caps™ ACID-RESISTANT CAPSULES have been used to protect the active microbiota from gastric juice, but initial studies have shown poor protection.

To improve protection, a new acid enzyme resistant capsule was developed that preserved the microbiota well in acidic medium and dissolved in alkaline medium. The study of the microbial composition by PCR with fluorescence detection confirmed that the number and composition of microorganisms remained unchanged after freeze-drying, indicating that their activity was preserved.

Conclusion: Our own studies have shown that it is possible to freeze-dry the substance of the monkey gut microbiota and produce capsules with enterosolubilised shells suitable for targeted delivery in fecal microbiota transplantation.

Keywords: Microbiome; FMT; Fecal microbiota transplantation; Gastrointestinal tract; Capsules

* Corresponding author: Alexandra Vadimovna Melikhova

1. Introduction

Fecal microbiota transplantation (FMT) may be an interesting alternative treatment method that can modify the composition of the gut microbiota and will promote energy extraction and synthesis of many nutrients, vitamins and metabolites. (Rinninella et al., 2019) [1]. Therefore, there is a need to develop convenient dosage forms for FMT. Members of the gut microbiota are microbial communities including archaea, bacteria and fungi [2]. All of them are not resistant to the effects of gastric juice. Therefore, systemic administration and local delivery are important ways of transplantation of gut microbiota [3]. The preparation of any solid dosage form requires dry microbial substance.

The freeze-drying (lyophilization) method is widely used in the production of biological drugs. In the Russian market, up to 99% of commercially available dry immunobiological preparations in ampoules, vials, powders, tablets and capsules are produced directly using the freeze-drying method [4]. The freeze drying process consists of three steps: 1) freezing of the material; 2) sublimation of ice and removal of the resulting vapors (removal of unbound moisture); 3) removal of bound moisture at temperatures above 0°C (freeze drying). The specificity of the process determines its main advantages: thermal inactivation of the material is excluded; minimal changes in the physicochemical and biological properties of the drug are ensured not only in the process of dehydration, but also during its subsequent long-term storage; the possibility of obtaining a dry product in prepackaged and sterile form (ampoules, vials) is facilitated [5,6].

The freeze-drying method was used to prepare and freeze-dried preparation of monkey intestinal microbiota substance, the active ingredient for the manufacture of dosage form in the form of capsules.

Dosage forms in the form of acid-fast capsules are widely used nowadays. But all these pharmaceutical developments are created exclusively for humans, taking into account their physiological characteristics and the activity of the gastrointestinal tract environment. Therefore, the question arises about the possibility of using conventional acid-resistant capsules in the treatment of monkeys and the method of their possible modification. To ensure targeted delivery to the small intestine, the capsules were additionally coated with enterosolubilised shell.

1.1. Objective of the study

To evaluate the feasibility of freeze-drying the substance of the monkey gut microbiota and the use of serial acid-tolerant capsules for the preparation of a finished form for fecal microbiota transplantation.

2. Materials and methods

2.1. Animals

Monkeys of the rhesus macaque species (*Macaca mulatta)* aged from 1 to 5 years, kept in the Kurchatov Complex of Medical Primatology (KCMP) of SRC "Kurchatov Institute", Sochi, were used in this work.

Monkeys were kept in a special room, kept in individual cages (equipped with feeders and automatic drinkers) with indication of animal numbers and groups. The ambient air temperature in the house for keeping monkeys was 21 - 28 °C, relative humidity 40 - 70 %; natural length of daylight hours. The food ration of animals is balanced in proteins, fats and carbohydrates, monkeys received pelleted feed, bread, fruits, according to the established norms.

2.2. Preparation of liquid substance for FMT

Liquid substance for FMT was prepared as follows: monkey faeces were homogenised in sterile phosphate buffer (PBS) containing 5% sucrose as cryoprotectant at a ratio of 1:4, respectively. The homogenisate was centrifuged for 5 min on a 300G centrifuge. The supernatant was separated and the resulting suspension was poured into 25-30 ml vials (layer height 7-15 mm). The vials were frozen at -70°C for storage and further lyophilic drying.

2.3. Production of an experimental sample of a dosage form for FMT

The frozen substance was kept at -70°C for at least a day. Then vials with the frozen substance were placed in the sublimator of the drying unit. Drying of microbial substance for FMT was carried out in the laboratory freeze-drying unit FreeZone 6 Labconco (USA), by convective heat supply to the frozen material through the plate on which the dehydrated material in vials was placed.

At the end of the dehydration process, the dried substance was extracted from the vials and crushed. The residual moisture content of the dried preparation ("loss in mass during drying") was determined by a widely used gravimetric method. The crushed dry preparation was used as active substance to fill capsules for FMT using a PRESSORE 100 Perni MC2 capsule machine.

The contents of the obtained dosage form (capsules) were analysed for microbial composition.

DR caps™ ACID-RESISTANT CAPSULES were used in this work. In vitro disintegration test of capsules in laboratory conditions was carried out in accordance with OFS.1.4.1.0005.14 P 1.2.1.2 in acidic and alkaline media.

To determine the protective effect of the capsules against gastric contents of monkeys in vivo, the capsules were filled with the medical radiopaque preparation Bar–VIPS.

2.4. Preparation of fecal slurry

0.8 ml of sterile isotonic sodium chloride solution was added to microcentrifuge tubes (1.5 ml volume). In each tube, 0.1 ml of feces was introduced with a separate tip (or disposable spatula) and thoroughly resuspended on a vortex until a homogeneous suspension was formed.

2.5. Preparation of the bacterial fraction

Freshly prepared fecal suspension was used. Tubes with suspension (watery feces) were centrifuged at 12000 g for 5 min on a MiniSpin centrifuge, Eppendorf, Germany. A bacterial fraction of 0.1 ml (the upper white–yellow part of the resulting sediment) was taken from each tube with a separate tip. The selected portion of the sample containing a high concentration of bacteria was transferred to a new 1.5 ml microcentrifuge tube. The sample was stored at –20°C for one week, at –70°C for a longer period of time.

2.6. PCR and DNA extraction

Nucleic acids were isolated from fecal samples using the reagent kit "DNA–sorb–B" produced by AmpliSens, FBUN Central Research Institute of Epidemiology, Rospotrebnadzor, in accordance with the manufacturer's instructions.

The reagent kit "COLONOFLOR-16 (biocenosis)" with detection on a CFX–96 (BioRad) instrument was used to detect 23 indicators (21 microorganism groups/species, total bacterial count and anaerobic imbalance), according to the manufacturer's instructions.

Bacterial diversity of gut microbiota was determined by qPCR using the reagent kit "COLONOFLOR-16 (biocenosis)" (AlfaLab LLC, Russia) on a CFX96 amplifier (Bio-Rad Laboratories Inc., USA).

Molecular genetic studies of liquid substances of intestinal microbiota collected from donors and freeze-dehydrated substances were carried out in accordance with the guidelines "Organisation of work of laboratories using nucleic acid amplification methods when working with material containing microorganisms of pathogenicity groups I-IV" MU 1.3.2569–09, Moscow, 2009 [7].

2.7. Radiological studies

In the course of the radiological study, conducted using a portable veterinary X-ray device Porta 100HF ("JOB Corporation", Japan), the protective properties of acid-resistant DR caps™ capsules were evaluated during the passage through the gastrointestinal tract of monkeys and the same capsules, but reinforced with an additional enterosoluble shell, which consisted of two layers: first - of hydroxypropyl cellulose (cluccel LF NF), second - of acetate phthalate cellulose, beeswax and tween-20, with the following ratio of components in the shell, wt.%: hydroxypropyl cellulose - 2,6; acetate phthalate cellulose - 35,3; beeswax - 4,0; tween-20 - 8,0.

During the period of the experiment, the animals were under the supervision of a veterinarian. Introduction of gelatin capsules containing barium sulphate was carried out with full fixation of animals using a rotary spreader, a tongue spatula with a round hole and an introducer (veterinary tablet dispenser). To exclude mechanical damage and to facilitate swallowing, 10 ml of water was injected into the oral cavity of the animals with a syringe. The capsules were administered on an empty stomach and after a meal. The exposure time of the capsules in the stomach ranged from 20 to 100 minutes. For immobilisation, animals were subjected to combined anaesthesia. Monkeys were administered a dose of Xyl 0.1 and Zoletil 0.05 ml/kg based on weight. Animals were transported in cages–carriers to

the manipulation room for radiography. Radiographs of the gastrointestinal tract of the monkeys were obtained in two projections. The animals were then placed in individual cages where they were withdrawn from anaesthesia.

3. Results and discussion

During the experiment the thermogram of the freeze-drying process was obtained, presented in FIG.1., in which the initial temperature of the shelves in the sublimator was -40° C, and the duration of the first stage of the process before the start of heating the shelves was 14 hours. Further, the temperature of the sublimator shelves was gradually raised every hour by 5° C. After completion of free moisture sublimation process (the second stage of drying), which was indicated by the rise in temperature of the material above 0° C, the shelf heating temperature was raised to 35° C. The third stage of the drying process (additional drying) was carried out for another 6 hours. The whole process of sublimation dehydration was 32 hours.

Figure 1 Temperature schedule for drying the substance of the monkey gut microbiota.

Dosage forms in the form of acid-resistant capsules are widely used nowadays. The pharmaceutical market offers a wide range of capsules made of different materials. We used commercially available DR caps™, which fully comply with pharmacopoeial requirements (OFS.1.4.1.0005.14 P 1.2.1.2).

However, as a result of the first studies, it was found that these capsules do not provide adequate protection of the active ingredient of the dosage form when passing through the gastrointestinal tract of experimental monkeys.

To clarify the protective properties of DR caps™ capsules, clinically healthy animals were injected with the barium sulfate-loaded capsules and radiological examination was performed.

Radiographs of the gastrointestinal tract of the monkey were obtained in the position of the animal lying on its back and on its side in an anterior straight projection every 10 minutes after giving the capsules until the appearance of a "plume" of barium sulphate from the destroyed capsules.

Absence or presence of protective effect of capsules from gastric contents was determined by the zone of capsule disintegration. Appearance of barium sulfate “plume” from broken capsules in the small intestine would mean the absence of optimal protective effect of these capsules.

Gastrointestinal radiographs of the monkeys taken during this experiment are shown in FIG. 2.

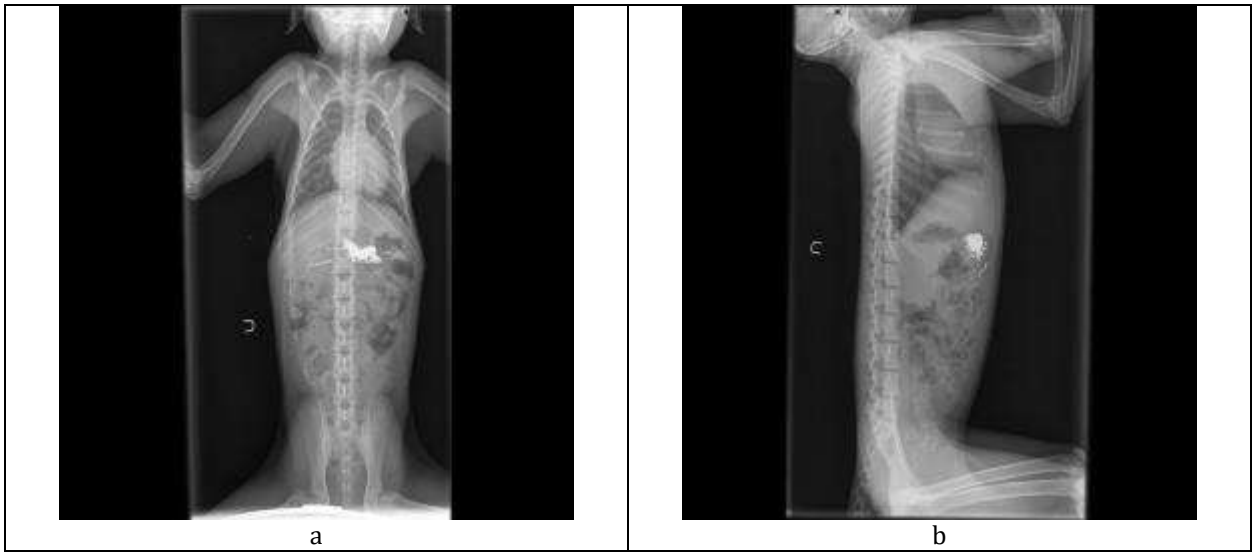

Figure 2 Gastrointestinal radiographs of the monkeys after administration of capsules without additional protective coating: (a) projection lying on the back; (b) projection lying on the side. Radiographs in two projections of animal MR #47724 m, which received barium sulphate capsules without additional protection, are presented. The projections show that the capsules completely disintegrated in the monkey's stomach ("plume" of barium sulphate), which cannot be recognised as satisfactory.

Once the dosage form enters the intestine from the stomach, the active ingredients should be released in the duodenum within 5–30 minutes, since digestion by pancreatic enzymes and absorption of metabolites mainly occur in the upper intestine.

Two synthetic polymer solutions were prepared to impart the required properties to the acido– enzyme-resistant coating:

- - 2% solution of hydroxypropyl cellulose LF NF;
- - of acetate phthalate cellulose, beeswax and tween-20 at the following ratio of components in the shell, wt.%: hydroxypropyl cellulose - 2,6; acetate phthalate cellulose - 35,3; beeswax - 4,0; tween-20 - 8,0.

The solutions were applied to the capsules in layers, alternating between each other, first with the first solution, then after complete drying - with the second solution. Since the capsules contained microbiological substance, the capsules were dried at +5° C.

To test the protective properties of the developed enterosoluble coating, several capsules without substance were coated with it. In vitro degradability testing of enteric-soluble capsules was performed according to OFS.1.4.1.0005.14 P 1.2.1.2 in 0.1 M hydrochloric acid solution for 2 hours. Intestine-soluble capsules were required to remain intact in 0.1 M hydrochloric acid solution for at least 1 h. Capsule damage was defined as any disruption of the capsule wall integrity that allowed the capsule contents to escape into the environment. The capsules were then rinsed with distilled water, placed in phosphate buffer solution with a pH value of 6.8 and exposed for 1 h. The temperature of the dissolution medium was monitored throughout the study and was (37 ± 0.5) °C. The rotation speed of the stirrer blades was 100 rpm.

As a result of laboratory tests the following was shown. When the capsules were exposed to 0.1 M hydrochloric acid solution for 3 h, the surface of the coating remained unchanged. In 15 minutes after placing the capsules in phosphate buffer solution, bubbles began to form on the surface of the enterosoluble coating, characterising a breach of the coating layer integrity (air enclosed inside the capsule came out).

The methodology of the following experiment was similar to that of the above experiment using acid resistant barium sulphate capsules.

The monkey received 2 DR caps™ size 2 capsules with a 2–x layer protective acid-fast coating. After 120 minutes of exposure, one capsule was in the stomach and the second one was in the small intestine. Both capsules showed defects in the protective coating. However, the defects in the capsule in the stomach are small and have occurred recently, as

single smears of contrast are detected in the surrounding space next to the capsule (upper capsule, FIG. 3.). The beginning of capsule destruction in the monkey's intestine is clearly observed, manifested by a change in its shape and release of contrast agent to the outside (lower capsule, FIG. 3).

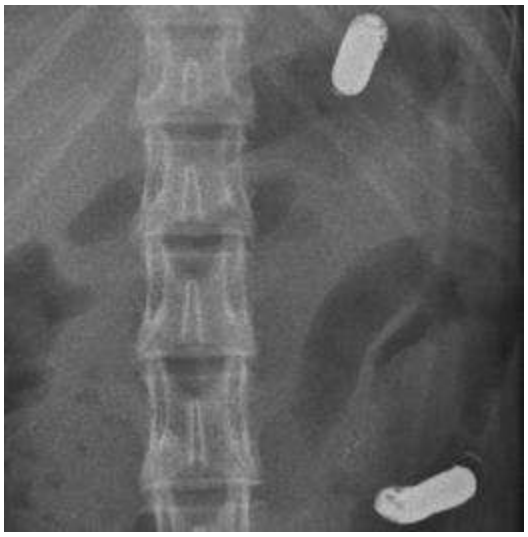

Figure 3 Radiograph of a monkey that received 2 capsules with additional protective coating (exposure 120 minutes).

When preparing capsules for FMT as described above, the composition and concentration of microorganisms contained in the selected gut microbiota should be preserved as much as possible.

For this purpose, the composition and number of microorganisms in the substance were determined after collection of biological material and after preparation of the preparation. The study was performed by qPCR with fluorescence detection.

The table shows the composition of microorganisms in the liquid substance and the same substance after freeze-drying.

Table 1 Quantitative composition of microorganisms of the liquid and dry substance of monkey gut microbiota**.**

No. n/a	Indicator	Liquid substance	Freeze-dried substance	Norma
1	Total bacterial mass	6×10^{8}	5×10^{9}	No more than 10^{12}
2	*Lactobacillus* spp.	2×10^{8}	2×10^{10}	10^{7} - 10^{8}
3	*Bifidobacterium* spp.	1×10^{10}	1×10^{9}	10^{9} - 10^{10}
4	*Escherichia coli*	6×10^{6}	1×10^{7}	10^{7} - 10^{8}
5	*Bacteroides* spp.	6×10^{8}	5×10^{9}	10^{9} - 10^{12}
6	*Faecalibacterium prausnitzii*	6×10^{8}	3×10^{8}	10^{8} - 10^{11}

Microorganisms of species *Bacteroides thetaiotaomicron*, *Akkermansia muciniphila*, *Enterococcus* spp., enteropathogenic *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Candida* spp, *Staphylococcus aureus*, *Clostridium difficile*, *Clostridium perfringens*, *Proteus vulgaris/mirabilis*, *Citrobacter* spp., *Enterobacter* spp., *Fusobacterium nucleatum*, *Parvimonas micra*, *Salmonella* spp. and *Shigella* spp. were not detected.

From the data presented in the table, it can be seen that in the liquid microbial substance in donors no disorders of microbiocenosis of the large intestine were revealed. The ratio of *Bacteroides fragilis* group/ *Faecalibacterium prausnitzii* was 0.1/0.01-100. After the microbial suspension was subjected to freeze dehydration, the same ratio was 16.7/0.01-100. The concentrations of probiotic microorganisms, as can be seen from the data obtained, increased after drying by 1-2 orders of magnitude.

Preparation of a dosage form for FMT in the form of capsules requires obtaining a dry active substance of gut microbiota. The study showed that the use of accelerated heating of the material at the pre-drying stage (stage 3 of the process) allowed to reduce the total duration of the process by 4 hours. Perhaps, in the future it is advisable to consider another, more intensive variant of the process realisation, for example, with the beginning of heating of the material at the stage of ice sublimation (2nd stage of the process). However, it is necessary to take into account the peculiarity of such a drying process, namely, massive removal of vapours of moisture sublimating from the material at increasing the range of shelf heating temperature and maximum capacity of the condenser providing freezing of these vapours on its surface.

The process of freeze drying of the liquid substance had no negative effect on either qualitative or quantitative composition of microorganisms (Table), providing the possibility of creating a dosage form in the form of hard gelatin capsules. The increase in the concentration of microorganisms per unit mass of the substance after drying is simply explained by the increase in its dry matter (i.e. dry microorganisms) due to the removal of moisture.

The results of the above experiment (FIG. 2.) showed the necessity of additional protection of the selected acid-resistant DR caps™ capsules by our specially developed acido-enzyme-resistant enterosoluble (acid-enzyme-resistant enteric soluble) coating. The action of acido–enzyme-resistant and enteric-soluble coating is due to the different properties of gastric and intestinal media due to different acidity and enzyme composition.

DR caps™ ACID-RESISTANT CAPSULES, additionally coated with enterosolubilised coating, withstand integrity for 3 hours in an environment simulating gastric juice. The developed coating fully meets all the specified characteristics, namely stable in acidic medium and soluble in alkaline medium.

In vivo experiments on monkeys confirmed the effectiveness of an additional protective acid-resistant coating based on hydroxypropylcellulose and acetate phthalate cellulose (FIG. 3), resistant to gastric juice, which is a prerequisite for the possibility of targeted delivery of the active ingredient into the intestine of monkeys.

3.1. Limitations of the study and directions for further research

The current study opens the door to further investigate the use of gut microbiota as an alternative treatment or to prevent the development of a number of diseases associated with altered gut microbiota.

4. Conclusion

The development of an oral method of FMT is impossible without an appropriate solid dosage form, which in turn requires a dry microbial substance. In our research for obtaining a dry preparation we used an intensified method of freeze dehydration with a duration of 32 hours, which provided preservation of both microbial diversity and concentration of microorganisms of the gut microbiota. Tests under laboratory conditions confirmed the possibility of using DR caps™ modified with a specially developed acid-resistant, enterosoluble coating that maintains integrity for 3 hours in a medium simulating gastric juice and dissolves for 15 minutes in a medium simulating small intestinal secretion. Experiments in monkeys confirmed that the capsules reached the intestine without disintegrating in the animal's stomach, demonstrating the ability to target the active ingredient of the dosage form to the intestine.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

Statement of ethical approval

All procedures performed with the participation of animals were in compliance with the ethical standards approved by the legal acts of the Russian Federation, the principles of the Basel Declaration and the requirements of the European Convention for the Protection of Vertebrate Animals Used for Experiments or Other Purposes, ETS №123 and Directive

№2010 /63/ EU, adopted by the European Parliament on 22 September 2010. Permission to conduct the work was obtained from the Bioethics Committee of FGBNU "Research Institute of Medical Primatology". The research protocol was approved by the Ethical Committee of the organisation (FGBNU "Research Institute of Medical Primatology") - №91 from 24.06.2022.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study

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