Regular Article

Effects of Simulated Weightlessness on Metabolizing Enzymes and Pharmacokinetics of Folic Acid in SD Rats

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Folic acid (FA) affects human physiology and drug metabolism. Up to now, the effect of microgravity on the pharmacokinetics of FA remains unclear. The pharmacokinetics of FA in Sprague–Dawley (SD) rats are laying a foundation for safe medicine administration of astronauts. Proteins expression of such FA metabolic enzymes as Methyltetrahydrofolate reductase (MTHFR), Cystathionine beta synthase (CBS) and Methionine synthase (MS) in a variety of organs was analyzed with Western-Blot, and mRNA expression was detected by RT-PCR. The plasma concentration–time profile of FA in normal or tail-suspended SD rats was acquired by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after oral administration of FA. Area under curve (AUC) and Cmax of FA in SD rats decreased significantly with extending period of tail-suspension. In terms of expressed level of metabolic enzymes over four suspension terms, as well as the level of the corresponding mRNAs, the following regularities were found: an obvious sharp decline of MTHFR tissue in kidney, a time-dependent increase of CBS in liver tissue and duodenum tissues, the resemblance of MS fluctuation to that of CBS in tested tissues. A four-week simulated microgravity of SD rats exhibits an unequivocal diminish of bioavailability of FA, and simulated microgravity shows a varying effect on the expression of FA-metabolizing enzyme in a variety of tissues.

Key words: folic acid; simulated weightlessness; metabolic enzyme; pharmacokinetics; tail-suspension model

INTRODUCTION

Nutrition plays a key role during space flights not only for the basic nutritive intake to meet the physical metabolic demands, but also for maintaining the organism in good health.1) Water-soluble vitamins, due to their limited endogenous storage, are of vital concern for space travelers. Folic acid (FA) is one of the essential B vitamins from foodstuffs (Fig. 1). Folate is the general term used to denote folic acid and those compounds exhibiting the activity similar to that of folic acid.2) Serving as coenzymes in many biological pathways, FA delivers 1-carbon unit in both amino acid reaction, nucleotide synthesis, and methylation reaction.3,4)

As with many nutrients, FA deficiency on an exploration mission can be catastrophic. Early space flight data show a reduction in red blood cell folate after long-duration missions.5) Folate levels are even more decisive during exploration missions than on International Space Station (ISS) regarding known increase in iron storage during long-duration spaceflight under exposure to ionizing radiation.6) The recorded data suggest that vision issues during spaceflight are associated with the folate and vitamin B-12 dependent 1-carbon transfer pathway.7)

Maintaining the nutrients of space food is critical for long-term manned spaceflights. Five food from the spaceflight food system have previously been evaluated for multi-year nutritional stability, and the results indicate that vitamin B1, B9 (folic acid), K, and C significantly degrade on a food-specific basis and may become inadequate during long-duration missions.8) Vitamins A, B12, B9, C, and E were retained at 70 to 95% levels for three years at 21°C in carefully formulated, fortified low water activity products. B9 degraded fastest among all the vitamins.9)

Folic acid requirements is known to be higher in life-stages with intense cell division, e.g., during pregnancy. Recommended folate intake for women of childbearing age is 400 µg daily. For adults the recommended daily intake is 400 µg.10) A minimum requirement of intake of FA is documented at 400 µg/d for astronauts during long-term spaceflight, without variation from ISS request.11)

As we know, spaceflight can alter human physiology as a result of such factors as fluid shifts, muscle and bone loss, immune system dysregulation, the changes in the gastrointestinal tract and metabolic enzymes. These alterations may affect the pharmacokinetics and/or pharmacodynamics of medications, thus the efficacy and safety of medications on astronauts.12)

It is evident that the folate level in the body changes during long-term missions, but it remains unknown if these changes are correlated to that of vitamin metabolism. Evaluations of folate metabolism during spaceflight may ensure the body’s health requirements, we investigate whether FA-metabolizing enzymes involved in one-carbon (1C) metabolism and the

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![Fig. 1. The Chemical Structure of Folic Acid](Image)
pharmacokinetics of FA vary under simulated weightlessness.

MATERIALS AND METHODS

Chemicals Folic acid and caffeine were purchased from National Institutes for Food and Drug Control. Methanol was purchased from Thermo Fisher Scientific (Rockford, IL, U.S.A.).

Animals and Their Treatment All the studies on animals were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals. The experimental protocol involving animals was reviewed and approved by the Institutional Animal Care and Use Committee of the Air Force Medical University. Four-week-old, healthy, specific pathogen-free, male Sprague–Dawley (SD) rats weighing 220 ± 20 g were supplied by the Experimental Animal Center of Air Force Medical University (Xi’an, People’s Republic of China). All rats were housed in internally flawless animal rooms at constant temperature (23 ± 2 °C) and humidity (55 ± 10%). The room was kept in a 12 h light/dark cycle and the rats had unrestricted food and water.

Drug Administration and Sample Collection To investigate the expression of FA-metabolizing enzymes in tissues, sixty-four SD rats were randomly divided into 4 control groups (CON-7 d, CON-14 d, CON-21 d, CON-28 d), 4 tail-suspension groups (SUS-7 d, SUS-14 d, SUS-21 d, SUS-28 d). Animals in all eight groups underwent fasting during the last 12 h of treatments and were anesthetized with pentobarbital sodium (40 mg/kg intraperitoneally (i.p.)) and killed by exsanguination via abdominal aorta. The liver, kidney, lung, duodenum, jejunum and ileum samples were rinsed with physiological saline, and then stored at −80 °C after lyophilization prior to analysis.

In the pharmacokinetics study, forty SD rats were randomly divided into control group (CON), 4 tail-suspension groups (SUS-7 d, SUS-14 d, SUS-21 d, SUS-28 d). The suspension groups were tail-suspended to simulate microgravity according to Morey–Holton model.2) Medical adhesive tapes were attached to the rat’s tail and fixed on the top of the cage. The tilt angle was about 30° in relation to the horizontal. Rats can freely move in its cage with free access to food and water. For the pharmacokinetics experiments, control and simulated microgravity rats (n = 8 for each group) were fasted for 12 h with free access to water before dosing. After rats were orally administered with 0.5208 mg/kg FA, blood samples were collected into heparinized tubes at scheduled times (0.167, 0.333, 0.667, 1, 1.5, 2, 3, 4, 6, 8, and 12 h). Blank plasma was collected before dosing. Plasma was collected after centrifugation (6037.2 × g for 10 min, 4 °C) and stored (−20 °C) until further analysis.

Western Blotting Analysis Total proteins were extracted in tissues with cold radio immunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) supplemented with a phenylmethanesulfonfluoride (1 mmol/L: Sigma). Homogenates were subjected to centrifugation at (6037.2 × g for 10 min at 4 °C) and supernatant fluids were collected. Protein concentrations of the supernatant fluid extracts were determined by the Lowry method. Equal amounts of protein samples were separated by 10% (v/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane.

After washing, the membranes were incubated overnight at 4 °C with one of the following primary antibodies: rabbit polyclonal antibodies against methylenetetrahydrofolate reductase (MTHFR), cystathionine beta synthase (CBS) and methionine synthase (MS). β-Actin (anti-mouse immunoglobulin G (IgG), 1:10000; Boster Biological Technology Co., Ltd., Wuhan, China) was set as the internal reference. After further washing, the membranes were incubated for 1 h with corresponding horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG or anti-mouse IgG, 1:10000; ComWin Biotech Co., Ltd., Beijing, China). Immunoreactive bands were visualized using an enhanced chemiluminescent substrate (Thermo Fisher Scientific) with a GE ImageQuant LAS 4000 mini (GE Healthcare, Waukesha, WI, U.S.A.). The intensity of protein bands was quantitated using a Gel Doc XR System (Bio-Rad, Hercules, CA, U.S.A.).

RT-PCR Analysis Total RNA was extracted from the tissues using Trizion Kit. (Invitrogen) and measured using ultraviolet spectrophotometry. Measurements were repeated for verification. Primers were designed according to the mRNA sequences (GenBank) of MTHFR, CBS, MS and ACTIN. The PCR products were subjected to agarose gel chromatography (1%) to confirm purity and concentration. cDNAs were synthesized using a reverse transcription kit (Promega, Madison, WI, U.S.A.). The forward primer of the MTHFR gene was 5'-CAGGAGAGCTGCGGAGAA-3', and the reverse primer of the MTHFR gene was 5'-CTGCTGCCGTCAAC-3'. The forward primer of the CBS gene was 5'-GAAGCCCGAGACGATCA-3', and the reverse primer of the CBS gene was 5'-TCGCACTCACCCTTCT-3'. The forward primer of the MS gene was 5'-GCCGGAGACGATCA-3', and the reverse primer of the MS gene was 5'-TGATTCCGGCGACTGTC-3'. The forward primer of the β-Actin gene was 5'-CACCGCGAGATACACCCTT-3', and the reverse primer of the β-actin gene was 5'-CCCATACACACCC-3'. Primers were then applied in a quantitative assay in the presence of SYBR Green to perform fluorescent quantitative PCR. After the reactions were complete, the amplification and solubility curves of DNA were analyzed and the data were processed using a gel imaging system (Glyko, Novato, CA, U.S.A.).

Quantitative Analysis of FA in Plasma Aliquots (100 µL) of plasma were mixed with 360 µL of methanol, and an internal standard, caffeine (10 µL of 100 ng/mL mobile phase) was added. After centrifugation, the supernatants were evaporated to dryness. The residues were dissolved in 100 µL of mobile phase (methanol/water with 0.1% formic acid) and analyzed with liquid chromatography-tandem mass spectrometry (LC-MS/MS). The detection limit of FA was 1 ng/mL, phase A (Methanol) and phase B (water with 0.1% formic acid) in the ratio of 55:45.

LC-MS/MS Analysis LC-MS/MS analysis was performed on an API LC-MS/MS system. Chromatographic separation was achieved by a Shimadzu LC system with an Agilent C18 column (4.6 × 150 mm, 3.5 µm), kept at 30 °C, at 0.6 mL/min with phase A (Methanol) and phase B (water with 0.1% formic acid) in the ratio of 55:45. The mass spectrometer was operated in the positive ion mode with electrospray ionization. The ionization source variables were a capillary voltage of 0.55 kV, source temperature of 150 °C, and desolvation gas temperature of 450 °C, The MS/MS transitions (m/z)
Fig. 2. Western Blotting Was Used to Analyze CBS, MS, MTHFR in Different Organs of Rats under Normal Gravity and Simulated Weightlessness A (Lung); B (Liver); C (Kidney); D (Duodenum); E (Jejunum); F (Ileum). Values were expressed as means ± S.D. (n = 6). **p < 0.01 vs. CON group.
monitored were 442.0 > 295.1 for FA, and 195.2 > 137.9 for IS. The declustering potential values set for FA and IS were 63 and 58 V, respectively. The collision energies values set for FA and IS were set at 21 and 28 eV, respectively. Data were acquired by the Analyst software (Version 1.6, Applied Biosystems/MDSSCIEX).

**Pharmacokinetics Analysis** The concentrations of FA in plasma at different time points were expressed as mean ± standard deviation (S.D.). The plasma concentration versus time curve was plotted. The pharmacokinetic parameters were analyzed by non-compartmental method using DAS Version 3.0 (Drug And Statistics, Mathematical Pharmacology Professional Committee of China, Beijing, China). Data were analyzed by GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, U.S.A.) and expressed as mean ± S.D. The statistical significance of differences between groups was determined using one-way ANOVA analysis. Differences were considered significant at \( p < 0.05 \).

**RESULTS**

**Effects of Microgravity on FA-Metabolizing Enzymes**

FA-metabolizing enzymes (CBS, MS, MTHFR) protein expression was examined using Western blotting analysis on the lung (Fig. 2A), liver (Fig. 2B), kidney (Fig. 2C), duodenum (Fig. 2D), jejunum (Fig. 2E), and ileum (Fig. 2F) samples under normal gravity and simulated weightlessness. FA-metabolizing enzymes mRNA expression was examined using Real Time PCR on the lung (Fig. 3A), liver (Fig. 3B), kidney (Fig. 3C), duodenum (Fig. 3D), jejunum (Fig. 3E), and ileum (Fig. 3F) samples under normal gravity and simulated weightlessness. The results showed that microgravity has a significant impact on FA-metabolizing enzymes in different tissues, the variation of mRNA and protein expression of enzymes were the same (Figs. 2, 3).

There were no obvious changes in MTHFR mRNA and protein expression level in liver. Results showed a significant and sustained decline in MTHFR mRNA and protein expres-
The pharmacokinetic variables of FA are summarized in Table 1. Compared with control groups, area under curve (AUC)\(_{0–\infty}\), CLz/F, \(V_f/F\), and \(C_{max}\) of tail-suspension groups decreased significantly. From 7 to 21 d, no obvious changes were observed for the \(t_{1/2}\), but \(t_{1/2}\) of SUS-28 d group was increased significantly. After the first week of tail-suspension, MRT dropped sharply and then increased, which was due to response to stress.

**DISCUSSION**

This study evaluated the impact of microgravity on enzymes involved in one carbon metabolism and the pharmacokinetics of FA by using tail-suspension model.

1C metabolism, mediated by the folate cofactor, constitutes a basis for multiple physiological processes. These include biosynthesis (purines and thymidine), amino acid homeostasis (glycine, serine, and methionine), epigenetic maintenance, and redox defense. In the one carbon metabolism pathway, enzymes help by pulling a single carbon atom from a folate or other carbon donor and subsequently attaching it to an amino acid, thus converting it to a different amino acid.\(^{15}\)

The three major enzymes involved in one carbon metabolism are methylenetetrahydrofolate reductase (MTHFR), MS, and CBS. CBS mediates conversion of homocysteine to cystathionine. MS catalyzes the transfer of a methyl group from other carbon donor and subsequently attaching it to an amino acid, thus converting it to a different amino acid.\(^{15}\)

The plasma concentration–time course of FA under normal and simulated weightlessness are shown in Fig. 4. With the prolonging of tail-suspension time, \(T_{max}\) went back to normal after 28d tail-suspension. There were double peaks in SUS-14 d group, SUS-21 d group and SUS-28 d group.
developed measurable ophthalmic changes after flight. Preflight serum concentrations of Hcy and cystathionine, as well as mean in-flight serum folate, were correlated with magnitude of change in refraction. The biochemical differences observed in crewmembers with vision issues strongly suggest that their folate and vitamin B-12 dependent 1C metabolism was significantly affected by weightlessness.7)

MTHFR, CBS and MS have wide tissue distribution. Microgravity has significant impact on the expression of the three major enzymes in different tissues. The expression of protein and mRNA of enzymes in same tissue are consistent. The varying tendencies of the enzymes were different in the same organ. Liver is the primary organ for folate metabolism. For MTHFR, there are no obvious differences among normal gravity and tail-suspension groups at different times. The protein and mRNA expression of MTHFR and MS decreased under simulated microgravity. So the questions that will inevitably follow: how microgravity impacts on the pharmacokinetics of FA?

FA has been proved to be a very important nutrient during space flight. However, the pharmacokinetics of FA under microgravity has not been examined. Under simulated weightlessness, Cmax gradually reduced. The Cmax of SUS-21 d group reduced by 55 percent, compared with the normal gravity group. Microgravity slow down the absorption of FA, and the concentration in blood decreased. Microgravity decreased the $AUC_{0-\infty}, Vd/F$ of FA.

Physiologically based pharmacokinetics (PBPK) modeling is a mathematical modeling technique for absorption, distribution, metabolism, excretion (ADME) of synthetic or natural chemical substances in humans and other animal species. Application of PBPK modeling used in conjunction with in-vitro in-vivo extrapolation (IVIVE) of ADME data can provide a useful starting point to understand and extrapolate pharmacokinetics in cancer patients22) and explore the response of the human body on low-concentration supplementation of vitamin D under sunlight-restrictive conditions.23) As a result of these studies, we observed the PK changes of FA under simulated microgravity, it is possible to make some dosing recommendations of FA during spaceflight, but more information is necessary to predict with precision all of the human pharmacokinetic variations occurring in spaceflight via PBPK modeling.

**CONCLUSION**

In conclusion, this study demonstrated that simulated microgravity shows a varying effect on the expression of FA-metabolizing enzyme (MTHFR, CBS and MS) in a variety of tissues. The pharmacokinetics of FA has been evaluated for the first time under simulated microgravity. Microgravity has severely impacted FA absorption and bioavailability. It is necessary to predict the FA dosage during spaceflight with PBPK modeling in follow-up studies.

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**Conflict of Interest** The authors declare no conflict of interest.

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