Effects of Inadequate Folate Intake on the Onset and Progression of Hypertensive Vascular Injury

Kumiko Takemori\textsuperscript{1}, Takuya Matsuo\textsuperscript{2}, Toshiaki Watanabe\textsuperscript{3}, Shuhei Ebara\textsuperscript{1}, Takaaki Chikugo\textsuperscript{4} and Takashi Kometani\textsuperscript{1}

\textsuperscript{1}Department of Food Science and Nutrition, Faculty of Agriculture, Kindai University, 3327–204, Nakamachi, Nara, Nara 631–8505, Japan
\textsuperscript{2}Department of Arts and Sciences, Faculty of Medicine, Kindai University, 377–2, Ohno Higashi, Osaka-Sayama, Osaka 589–8511, Japan
\textsuperscript{3}Department of Health and Nutrition, Faculty of Health Science, Osaka Aoyama University, 2–11–1, Niïna, Mino, Osaka 562–8580, Japan
\textsuperscript{4}Department of Pathology, Faculty of Medicine, Kindai University, 377–2, Ohno Higashi, Osaka-Sayama, Osaka 589–8511, Japan

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Summary

We investigated the effects of inadequate folate intake on the onset and progression of hypertensive organ injury. In the present study, 5-wk-old male stroke-prone spontaneously hypertensive rats (SHRSP) were fed with a normal-folate (control; 160–170 µg of folate/100 g diet) or low-folate (8–10 µg of folate/100 g diet) diet until they reached 25 wk of age. After the animals reached 10 wk of age, the bodyweight of the rats in the low-folate group was lower than that of the rats in the control group. Regarding blood pressure, both groups had severe hypertension of \$230\text{ mmHg at 12 wk of age that was not significantly different between the groups. At 16 wk of age, the low-folate group had a low number of blood cell types. The folate levels in the serum, liver, and kidneys of these rats were significantly lower (}p<0.01\text{) and the serum homocysteine level in the low-folate group was significantly higher than in the controls. The low-folate group had a significantly lower testicular weight than the control group (}p<0.05\text{) and arterial hypertrophy, spermatogenesis arrest, and interstitial connective tissue hyperplasia were observed. However, there was no clear difference in lesions in other organs. These results indicated that under low folate status, SHRSP causes hematopoietic disorders and exacerbates hypertensive vascular injury at various degrees by organ type.

Key Words

low folate, hypertension, vascular injury, spermatogenesis arrest, testicular artery

We have investigated the pathophysiology of vascular injury using stroke-prone spontaneously hypertensive rats (SHRSP), an animal model of hypertension and its sequelae. We previously clarified that free radicals have an important role in the onset of vascular injury (1, 2) and investigated the effects of various dietary factors on the suppression of the onset of vascular lesions (3, 4). Although the antioxidant effects of vitamins E and C are well known as dietary factors related to the suppression of vascular lesions (5), the effect of the B complex vitamins on the development of hypertensive lesions is unknown. The Sakado Folate Project in Japan investigated fortification of the diet with folic acid to prevent arteriosclerosis, which causes dementia and stroke (6). Furthermore, folic acid and B vitamins are thought to suppress the onset of metabolic syndrome.

Recently, it was demonstrated that folate deficiency affected the onset of vascular injuries related to hyperhomocysteinemia (7). Folate is involved in DNA synthesis and is required as a coenzyme for methionine synthase that converts homocysteine to methionine. Hyperhomocysteinemia was reported to have a role in the onset of cardiovascular disorders by impairing nitric oxide production from vascular endothelial cells and by reducing vasorelaxation responses by increasing oxidative stress and reducing the antioxidative capacity (8). Lee et al. reported that supplementation of the diet with folic acid improved endothelial injuries of the brain microvessels (9). Furthermore, Fakouri et al. revealed that folic acid administration prevented testicular ischaemia-reperfusion injury (10).

Although these reports reveal the beneficial effects of folic acid, the effects of insufficient folic acid intake on hypertensive lesions remain unclear. Therefore, we used the SHRSP model, which causes severe hypertension and vascular injury in various organs, to investigate the effects of insufficient folic acid on its pathogenesis. Previous studies will suggest that folic acid deficiency significantly exacerbated hypertension and its sequelae in SHRSP. It is expected that elucidating the pathophysiology of SHRSP will demonstrate the importance of folic acid in the circulatory system and its effects in hematopoietic disorders.

E-mail: kuriman@nara.kindai.ac.jp
Low-Folate Environment and Vascular Injury

MATERIALS AND METHODS

Animals. Five-week-old male SHRSP rats (Kindai University, Osaka, Japan) were used and divided into two groups: control (n=10) and low-folate (n=10) groups of equal mean weight and blood pressure. Throughout the experiment, rats were housed individually in stainless-steel cages in an environmentally controlled room with a 12-h light/dark cycle at 23±1°C. The animal experiments were conducted with the approval of the Animal Research Committee, Kindai University (approval number: KAAG-28-010), and in compliance with the “Guidelines for Animal Experiments at the Faculty of Agriculture, Kindai University.”

Diets. A standard purified diet (AIN-93M; CLEA Japan Inc., Tokyo, Japan) was used. The compositions of normal-folate (control) and low-folate diets and the composition of the vitamin mix are shown in Tables 1 and 2, respectively. Succinyl sulfathiazole (Sigma-Aldrich Co. LLC., Saint-Louis, MO, USA) was added to the low-folate diet (11). The animals were allowed free access to food and drinking water.

Measurements of blood pressure and bodyweight. The rats were placed in an incubator (NK-210-3; Natsume Seisakusho Co., Ltd., Tokyo, Japan) set to 40°C and warmed for 10 min. The blood pressure measurements were taken by the tail-cuff method (BP-98A; Softron Co., Ltd., Tokyo, Japan) without anesthesia. The blood pressure measurement was performed once a week concurrently with bodyweight measurement.

Cytometry. Blood was collected from the tail vein using 5% tetrasodium ethylenediaminetetraacetic acid (Dojindo Laboratories, Kumamoto, Japan) with normal saline as an anticoagulant. Cytometry was performed using an automated hematology analyzer (XT-1800; Sysmex Co., Hyogo, Japan).

Sample collection and histological observations. Samples were collected from five rats per group at 16 wk of age and 25 wk of age for the remaining rats. Blood was collected under anesthesia using a combination of somnopentyl (Kyoritsu Seiyaku Corporation, Tokyo, Japan) and isoflurane (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The collected blood was left for 2 h at room temperature and centrifuged (9,100 × g, 10 min, 4°C) to obtain the serum. The brain, heart, kidneys, liver, and testicles were removed and weighed. The removed organs were fixed in 10% formalin buffer (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan), and paraffin-embedded blocks were prepared. Then, 5-μm sections were prepared from each block, stained with hematoxylin and eosin (HE), and observed using an all-in-one fluorescence microscope (BZ-X700; Keyence Corporation, Tokyo, Japan).

Folate levels in the serum and tissue and serum homocysteine levels. To quantify folate in the serum and tissue samples, we used a microbioassay that contained Lactobacillus rhamnosus (NBRC3425) (12). Serum homocysteine levels were measured by a commercially available kit (Homocysteine ELISA Kit, Cell Biolabs, Inc., San Diego, CA, USA).

Statistical analysis. Experimental data were expressed as the mean±standard deviation. The Student’s t-test or Welch’s t-test was used, depending on the results of the F-test, for the analyses of differences

### Table 1. Diet composition.

|                      | Control diet | Low-folate diet |
|----------------------|--------------|----------------|
| Folic acid           | 160–170 (μg/100 g) | 8–10 (μg/100 g) |
| Corn starch          | 61.0692 (%)   | 60.0692 (%)    |
| Casein (vitamin free)| 14.0000      | 14.0000        |
| Granulated sugar     | 10.0000       | 10.0000        |
| Microcrystalline cellulose | 5.0000       | 5.0000        |
| Soybean oil          | 4.0000        | 4.0000        |
| Mineral mix          | 3.5000        | 3.5000        |
| Vitamin mix without folic acid¹ | 1.0000 | 1.0000 |
| α-Corn starch        | 1.0000        | 1.0000        |
| Choline bitartrate   | 0.2500        | 0.2500        |
| t-Cystine            | 0.1800        | 0.1800        |
| tert-Butylhydroquinone | 0.0008      | 0.0008        |
| Succinyl sulfathiazole | —            | 1.0000        |

¹The composition of vitamin mix is shown as Table 2.

### Table 2. The composition of vitamin mix.

|                      |                |
|----------------------|----------------|
| Granulated sugar     | 97.6580        |
| Vitamin E            | 1.5000         |
| Nicotinic acid       | 0.3000         |
| D-Pantothenic acid calcium | 0.1600 |
| Biotin (2%)¹         | 0.1000         |
| Vitamin B₂ (80%)¹    | 0.0720         |
| Vitamin B₆           | 0.0700         |
| Vitamin B₁           | 0.0600         |
| Vitamin A (1,000,000 IU/g) | 0.0400 |
| Vitamin D₃ (500,000 IU/g) | 0.0200 |
| Vitamin B₁₂ (2%)¹    | 0.0125         |
| Vitamin K₁           | 0.0075         |

¹Active ingredient.

The ratio of the total feed is shown below.

Biotin: 2%×0.1%×1%=0.00002%.

Vitamin B₂: 80%×0.072%×1%=0.000576%.

Vitamin B₁₂: 2%×0.0125%×1%=0.0000025%.
Table 3. Blood cell count at 16 wk of age.

|                | Control (n=5) | Low-folate (n=5) |
|----------------|---------------|------------------|
| WBC (10^3/µL) | 43.7±11.9     | 13.5±1.9*        |
| NEUT (10^3/µL)| 10.0±7.2      | 2.8±0.2          |
| LYMPH (10^3/µL)| 31.7±8.4      | 10.8±1.1*        |
| MONO (10^3/µL)| 1.5±0.6       | 0.2±0.1*         |
| EO (10^3/µL)  | 0.5±0.2       | 0.2±0.1*         |
| BASO (%)       | 0.0±0.0       | 0.0±0.0          |
| NEUT (%)       | 21.5±12.5     | 19.9±1.6         |
| LYMPH (%)      | 74.1±12.6     | 77.5±1.4         |
| MONO (%)       | 3.3±0.8       | 1.4±0.6*         |
| EO (%)         | 1.1±0.2       | 1.2±0.5          |
| BASO (%)       | 0.0±0.0       | 0.0±0.0          |
| RBC (10^6/µL) | 956±45        | 764±48**         |
| HGB (g/dL)     | 14.7±0.5      | 12.7±0.6**       |
| HCT (%)        | 42.4±18.1     | 37.1±2.1**       |
| MCV (fL)       | 44.4±0.4      | 48.5±0.8**       |
| MCH (pg)       | 15.4±0.2      | 16.7±0.5**       |
| MCHC (g/dL)    | 34.8±0.4      | 34.3±1.2         |
| PLT (10^3/µL) | 51.3±3.7      | 42.5±14.3        |
| RDW-SD (fL)   | 25.7±0.4      | 28.6±1.0**       |
| RDW-CV (%)     | 19.1±0.2      | 17.6±1.2         |
| PDW (fL)       | 9.0±0.2       | 10.3±0.4**       |
| MPV (fL)       | 7.8±0.0       | 8.9±0.4**        |
| P-LCR (%)      | 11.1±0.7      | 18.2±2.3**       |
| PCT (%)        | 0.4±0.026     | 0.4±0.1          |

Mean±SD, *p<0.05, **p<0.01.
WBC: white blood cell count. NEUT: neutrophil count. LYMPH: lymphocyte count. MONO: monocyte count. EO: eosinophil count. BASO: basophil count. NEUT%: differential count of neutrophil. LYMPH%: differential count of lymphocyte. MONO%: differential count of monocyte. EO%: differential count of eosinophil. BASO%: differential count of basophil. RBC: red blood cell count. HGB: hemoglobin content. HCT: hematocrit level. MCV: mean corpuscular volume. MCH: mean corpuscular hemoglobin. MCHC: mean corpuscular hemoglobin concentration. PLT: platelet count. RDW-SD: red cell distribution width-standard deviation. RDW-CV: red cell distribution width-corpuscular-volume. PDW: platelet distribution width. MPV: mean platelet volume. P-LCR: platelet-larger cell ratio. PCT: plateletcrit.

Mean values for white blood cell count (10^3/µL; p<0.05), hemoglobin content (g/dL; p<0.01), and hematocrit level (%; p<0.01). However, the mean corpuscular volume (fL; p<0.01), mean corpuscular hemoglobin (pg; p<0.01), and red cell distribution width (fL; p<0.01) in the low-folate group were significantly higher than in the control group. Moreover, the low-folate group showed pancytopenia with lower values for white blood cell count (10^3/µL; p<0.05), lymphocyte count (10^3/µL; p<0.05), monocyte count (10^3/µL; p<0.05), eosinophil count (10^3/µL; p<0.05), and percentage of monocytes (%; p<0.05). However, the low-folate group showed significantly higher platelet distribution width (fL; p<0.01), mean platelet volume (fL; p<0.01), and platelet-large cell ratio (%; p<0.01) compared with the control group.

RESULTS

Changes in the blood pressure, bodyweight, and food intake of rats in the low-folate group

Figure 1 shows the changes in blood pressure, bodyweight, and food intake in the groups. Regarding blood pressure, both groups had severe hypertension (>230 mmHg) from 12 wk of age (Fig. 1A). From 10 wk of age, the bodyweight of the low-folate group rats remained low; then it started to decrease rapidly at 23 wk of age, and at 24 wk, four out of the five rats died naturally (Fig. 1B). The low-folate group had a lower food intake than the control group from 20 wk of age onwards (Fig. 1C).

Blood cell count

Table 3 shows the results of cytometry at 16 wk of age. Compared with the control group, the low-folate group had significantly lower red blood cell counts (10^6/µL; p<0.01), hemoglobin content (g/dL; p<0.01), and hematocrit level (%; p<0.01). However, the mean corpuscular volume (fL; p<0.01), mean corpuscular hemoglobin (pg; p<0.01), and red cell distribution width (fL; p<0.01) in the low-folate group were significantly higher than in the control group. Moreover, the low-folate group showed pancytopenia with lower values for white blood cell count (10^3/µL; p<0.05), lymphocyte count (10^3/µL; p<0.05), monocyte count (10^3/µL; p<0.05), eosinophil count (10^3/µL; p<0.05), and percentage of monocytes (%; p<0.05). However, the low-folate group showed significantly higher platelet distribution width (fL; p<0.01), mean platelet volume (fL; p<0.01), and platelet-large cell ratio (%; p<0.01) compared with the control group.

Organ weights

Table 4 shows the comparison of organ weights at 16 wk of age. No difference was found in organ weights (brain, heart, kidneys, and liver) between the groups. The bilateral testicles of the low-folate group had a significantly lower weight than those of the control group (p<0.05).

Histological findings

Figure 2 shows the results of histological analysis.
using HE staining. Numerous sites of vascular hyper trophy and a smaller number of sites of glomerular necrosis and vascular necrosis in the kidneys of both groups were observed (Fig. 2A and B). Mild centrilobular cell degeneration and necrosis were observed in the liver of one rat in the control group, whereas degeneration and necrosis of hepatic cells surrounding the central vein as well as sinusoidal dilatation with hepatic cord atrophy were present in rats in the low-folate group (Fig. 2C and D). The low-folate group had marked testicular atrophy, with spermatogenesis arrest and numerous arteriole necroses (Fig. 2E and F). No remarkable changes were found in the brain and heart samples between groups.

Comparison of folate levels in serum and tissue samples, and serum homocysteine levels

Figure 3 shows the serum, renal, and hepatic folate levels and serum homocysteine level. The folate content in the low-folate group was significantly lower in the serum (Fig. 3A, as well as in the liver and kidneys (Fig. 3B, \( p < 0.01 \)) compared with controls. However, the serum homocysteine level was significantly higher in the low-folate group than in the control group (Fig. 3C, \( p < 0.01 \)).

DISCUSSION

In addition to anemia and neural tube defects, folate deficiency is involved in the onset of vascular injury (13). The present study investigated the effects of low folate intake on the onset and progression of hypertensive organ disorders using SHRSP rats, which spontaneously develop severe hypertension and sequelae. The SHRSP group had a marked increase in blood pressure without any artificial treatment, which was age dependent and led to severe hypertension >230 mmHg. SHRSP rats were reported to develop severe vascular injuries in the cerebral, cardiac, and renal vessels as well as testicular and mesenteric arteries (14).

The bodyweights of the low-folate group rats remained lower than those of the control group rats from 10 wk of age, which reached statistical significance after 18 wk of age (\( p < 0.05 \)). Folate deficiency induces disorders of the hematopoietic system (15) and at 16 wk of age, pancytopenia appeared in the low-folate group. In addition, the low-folate group rats had significantly higher mean corpuscular volume and mean corpuscular hemoglobin compared with the control group (\( p < 0.01 \)), but no difference was found for MCHC. In contrast, hemoglobin content (\( p < 0.01 \)) in the low-folate group was lower than in controls indicating the low-folate group was in a state of serious anemia.

In humans, inadequate folate intake might be a risk factor for the occurrence of hypertension (16). In addition, a study of SHR demonstrated that folate deficiency caused hypertension related to elevated oxidative stress and insulin resistance (17). In the present study using SHRSP rats, both groups had similar severe hypertension (approximately 230 mmHg) at the age of 12–16 wk. Despite the significantly lower folate content in the low-folate group compared with the control group
Arterial hypertrophy and tubular atrophy, as well as some arterial necrosis in kidney samples, were observed in both groups. Furthermore, no stroke lesion was observed in either group. One possible contributor to these unexpected results is the effect of diet composition. This incidence of stroke was 98% among SHRSP rats fed a Funabashi SP Diet (Funabashi Farm Co., Ltd., Chiba, Japan) (14). The protein source in Funabashi SP Diet is fish powder with 1% salt added to the diet. In the current study, casein protein was used as the protein source in the diets, which were based on the formulation of AIN-93. It was reported that a difference in diet composition affected the incidence of hypertensive sequelae as well as the severity of the lesion (18). This might explain why lesions in the kidneys and brains in the current study did not increase in severity.

The low-folate group had a significantly lower testicular weight than the control group as well as seminiferous tubular atrophy, arteriole wall hypertrophy, and necroses as assessed by histology. The low-folate group also had markedly reduced numbers of spermatogenic cells, anisokaryosis of the spermatogenic cells, and interstitial fibrosis. It was reported that folate excess or deficiency caused reduced DNA methylation (19) and spermatogenesis arrest (20). These studies suggest the importance of replenishing the body with an appropriate amount of folate. In the present study, vascular wall hypertrophy in the testicular artery was observed in the control SHRSP group. Interestingly, testicular arterial necrosis appeared only when severe hypertension coexisted with a low-folate environment. In preliminary experiments, anemia and testicular atrophy were observed in Wistar–Kyoto rats with normal blood pressure that were fed a low-folate diet. However, no abnormalities in the testicular artery were found (data not shown).

Histological analysis of the liver demonstrated fatty degeneration in the low-folate group. During the state of chronic folate deficiency, induction of a choline
Low-Folate Environment and Vascular Injury

...metabolism disorder was attributed to the occurrence of abnormal one-carbon transfer in the liver that induced hepatic steatosis (21). This might explain the liver observations in the present study. Gao et al. reported a homocysteine concentration <10 μmol/L and blood pressure of approximately 190 mmHg in untreated SHR. However, in rats intravenously administered homocysteine, the blood homocysteine concentration increased 3-fold and the blood pressure was increased (22). The serum homocysteine concentration in control SHRSP in our study was 40.5 ± 2.2 μmol/L, which was higher than the above-mentioned report in SHR. The reason for this is unknown, but it might be related to sex differences, age, and breeding environment. Although the homocysteine level was higher in the low-folate group than in the control group, high blood pressure led to similar severe hypertension (>230 mmHg) in both groups. Even at a low dose, folic acid reduced plasma homocysteine and attenuated hyperhomocysteinemia-induced glomerular injury by inhibiting oxidative stress and apoptosis (22). This supports the idea that the aggravation of hypertensive damage is related to increased homocysteine because of folic acid deficiency. It was recently reported that homocysteine signals through angiotensin II receptors to exacerbate disorders attributed to aneurysmal vascular necrosis (23). Therefore, interactions between folate metabolism and the renin–angiotensin system might exacerbate vascular necrosis. We already revealed that the renin–angiotensin system has a role in the onset of vascular injury in SHRSP (1). Furthermore, Zhu et al. reported that the concomitant administration of losartan and folate to SHRSP improved alterations in the aortic endothelial structure without changing the homocysteine level. However, the mechanism of action underlying this process is yet to be elucidated (24). A review of a study by Azu in SHRSP rats revealed that a structural alteration in the vascular wall of the testicles during a state of hypertension and decreased blood flow contributed to spermatogenesis arrest (25). In the testicles of SHRSP rats, the coexistence of inhibited DNA synthesis, anemia, and severe hypertension under a low folate environment causes severe spermatogenesis arrest.

Zhang et al. reported that feeding SHRSP rats with a high-methionine diet increased the blood pressure and serum homocysteine levels as well as reducing inflammatory responses. These conditions were improved by adding vitamins B6 and B12 to folate in the diet (26). Therefore, a deficiency of folate or vitamins involved in folate metabolism might contribute significantly to the progression and exacerbation of underlying diseases, including hypertension, arteriosclerosis, and diabetes.

Unexpectedly, no exacerbation of hypertensive vascular lesions related to folic acid deficiency was observed, except in the testicles. Although the cause was not elucidated in this study, our results suggest that the occurrence of vascular lesions related to folic acid deficiency cannot be explained by homocysteine alone, and that it probably depends on the presence or absence of underlying disease and its severity. In the future, we will examine the interrelationship between methionine metabolism-related nutrients and the pathophysiology of vascular injury and systemic organs in more detail. We hope this study will contribute to the acquisition of basic data to prevent the occurrence of vascular disorders by supplementing the diet with an appropriate amount of B complex vitamins.

Authorship
Research conception and design: KT and TM; experiments: KT and TM, SE, and TC; statistical analysis of the data: KT and TM; interpretation of the data: TW and TK: writing of the manuscript: KT and TM.

Disclosure of state of COI
No conflicts of interest are declared.

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