Original article (DINT-D-14-00038) revised

Folic acid supplementation of aspirin therapy further improves vascular endothelial function among patients with type 2 diabetes: a short-term crossover study

Shuichi Sato, Yuji Tajiri, Hitomi Nakayama, Kentaro Yamada

Division of Endocrinology and Metabolism, Department of Medicine, Kurume University School of Medicine, Kurume 830-0011, Japan.

Address for correspondence: Sato S. Division of Endocrinology and Metabolism, Department of Medicine, Kurume University School of Medicine, 67 Asahimachi, Kurume 830-0011, Japan.
Phone: +81-942-31-7563
Fax: +81-942-35-8943
E-mail: dem_sato@med.kurume-u.ac.jp

Short running title: Folic acid and aspirin therapy

Abstract

Aim To examine the effects of supplementing aspirin therapy with folic acid on vascular endothelial function among patients with type 2 diabetes.

Methods This was a randomized open label crossover study. Fifteen patients with type 2 diabetes (group A) were first treated with 100 mg/day aspirin plus 20 mg/day folic acid for 1 week and then received aspirin alone during the second week. Conversely, 17 patients (group B) received aspirin alone during the first week and received the combination therapy during the second week. Fourteen patients (group C) received folic acid alone for one week. Flow mediated dilatation (FMD) was examined at baseline and after each treatment.

Results In group A, the patients' FMD levels increased from 3.7 ± 2.0 % to 7.2 ± 3.1 % during the first week and decreased to 5.9 ± 2.8 % after the second week. In group B, FMD levels increased from 5.1 ± 2.2 % to 6.8 ± 2.9 % during the first week and further increased to 9.0 ± 3.6 % after the second week. In group C, FMD levels increased from 5.2 ± 3.3 % to 6.9 ± 3.8 %. Concomitantly, serum homocysteine (Hcy) levels decreased during folic acid administration in group C, but not in groups A and B. Addition of folic acid did not significantly affect levels of interleukin-6 or high sensitivity C-reactive protein.

Conclusion Supplementation of aspirin therapy with folic acid resulted in further improvement in endothelial function. The mechanism of the beneficial effect remains to be elucidated, although it is unlikely to be mediated by suppression of Hcy production or attenuation of chronic inflammation.

Key words: Endothelial function, Flow-mediated dilatation (FMD), Aspirin, Folic acid, Homocysteine, Type 2 diabetes mellitus

Introduction

Type 2 diabetes is a major risk factor for cardiovascular diseases (CVD). Putative mechanisms of these vascular complications include inflammation of the vascular wall, oxidative stress, and the exacerbation of platelet aggregation because of hyperglycemia or other metabolic disorders of the diabetic state. Vascular endothelial dysfunction has been recognized as an early stage of arteriosclerosis because the endothelium is crucially important in maintaining both vascular tone and the normal structure of blood vessels [1–3]. One endothelium-derived vasoactive mediator of major importance is nitric oxide (NO), which is formed from the amino acid precursor L-arginine and endothelial nitric oxide synthase (eNOS) [4]. A deficiency in NO production may be involved in endothelial dysfunction [5].

Platelet aggregation is enhanced in the diabetic state [6], and administration of low-dose aspirin has been recommended to prevent thrombosis in diabetic patients [7, 8]. Endothelial dysfunction can be improved by low-dose aspirin therapy, suggesting that platelets regulate vascular NO bioactivity [9]. Aspirin may also exert its beneficial effect on endothelial function through its anti-inflammatory properties [10, 11].

Folic acid, a water soluble vitamin, increases NO production by stabilizing tetrahydrobiopterin (BH4), a coenzyme for eNOS [12, 13]. The active form of folic acid, 5-methyltetrahydrofolate, has direct effects on the enzymatic activity of NO synthase both in recombinant eNOS and in cultured endothelial cells. Furthermore, folic acid reduces serum levels of homocysteine (Hcy), which is a potent excitatory neurotransmitter that binds to the Nmethyl-D-aspartate receptor and leads to oxidative stress, cytoplasmic calcium influx, cellular apoptosis, and endothelial dysfunction [14]. Although low-dose aspirin therapy is recommended for individuals with cardiovascular risk factors, there is concern that administration of aspirin may reduce serum folic acid concentration by acceleration of urinary folic acid excretion [15]. Furthermore, aspirin is rapidly and irreversibly converted into salicylic acid, which inhibits production of tetrahydrofolate [16], an important methyl donor for remethylation of Hcy into methionine [17]. Thus, folic acid supplementation in aspirin therapy is expected to have an additive or a synergistic effect for prevention of CVD. In this study, we investigated the effects of the combination therapy of aspirin and folic acid on endothelial function and other biological variables among patients with type 2 diabetes.

Subjects and methods

Subjects

We enrolled 46 patients with type 2 diabetes who had been admitted to Kurume University Hospital

for glycemic control (Table 1). Fifteen patients had been treated with sulfonylureas, 11 with metformin, 4 with pioglitazone, 14 with DPP-4 inhibitors, and 4 with insulin. The exclusion criteria included HbA1c[12.0 %, use of anti-platelet drugs, pregnancy and lactation, chronic inflammatory diseases, a recent acute infection, serum creatinine level [1 mg/dl, liver dysfunction (AST[40 IU/L or ALT[40 IU/L), known malignant diseases, current consumption of tobacco products, aspirin allergy, known history of CVD events, proliferative diabetic retinopathy, macro-albuminuria defined as a urinary albumin level [300 mg per day, and painful diabetic polyneuropathy.

Appropriate dietary management was continued throughout the study period. Subjects were required to avoid any vigorous exercise. Written informed consent was obtained from all subjects. The study design was approved by the ethics committee of Kurume University School of Medicine and was conducted according to the principles outlined in the Declaration of Helsinki.

Study design and materials

After a period of stabilization of such metabolic parameters such as blood glucose, blood pressure, and serum lipids for 7 ± 2 days (mean ± SD), the subjects were randomly assigned to group A, group B, or group C (Fig. 1). The subjects of group A were given 20 mg folic acid (Foliamin®, Nihon Pharmaceutical, Tokyo, Japan) plus 100 mg aspirin (Bayaspirin®, Beyer, Leverkusen, Germany) once daily during the first week and then aspirin alone during the second week. The subjects of group B were administered aspirin during the first week and received the combination therapy during the second week. The subjects of group C were given 20 mg folic acid for 7 days. Flow-mediated dilatation (FMD), body weight, and blood pressure were measured after an overnight fast, and blood was collected at baseline and at the end of each week.

Assessment of endothelial function

Brachial artery FMD, a surrogate marker for vascular endothelial function, was measured by use of a vascular ultrasound system equipped with an edge-tracking system and a pulsed Doppler flow velocimeter (Unex, Nagoya, Japan) [18], at the supine position, before breakfast in a quiet room, in accordance with international guidelines [19]. In brief, after measurement of the diameter of the brachial artery at rest, the cuff was inflated to 200 mmHg for 5 min. After prompt cuff deflation, the diameter of the artery was monitored continuously. FMD levels were calculated as follows:

FMD (%) = (maximum diameter－diameter at rest × 100 / diameter at rest.

Blood sampling and measurements

Blood samples were obtained from the antecubital vein. Glucose, HbA1c, triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured in accordance with standard procedures. Folic acid and interleukin-6 (IL-6) levels were determined by chemiluminescence enzyme immunoassay. Hcy levels were measured by HPLC (Hitachi, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed by use of JMP software version 10 (SAS Institute, Cary, NC, USA). To compare baseline values for each group, Student's t-test was used for continuous variables after confirming the normality of the data distribution by use of the Shapiro–Wilk normality test. Categorical variables were analyzed by use of the chisquared test. Results for more than two groups were compared by one-way, repeated-measures analysis of variance (ANOVA). The effects of drugs on FMD values were compared by using the ratio of post-treatment FMD to baseline FMD. The ratios for FMD, serum levels of folic acid, hsCRP, and IL-6 were logarithmically transformed to achieve a normal distribution before applying the t-test. All data are presented as mean ± SD, and a p-value<0.05 was considered to be statistically significant. When data in three groups were compared, a significant p-value was<0.017 (Bonferroni correction).

Results

In group A, patients' FMD levels increased from 3.7 ± 2.0 % to 7.2 ± 3.1 % ($p = 0.001$) during the first week and decreased to 5.9 ± 2.8 % ($p = 0.02$) after the second week (Fig. 2a). In group B, FMD levels increased from 5.1 ± 2.2 % to 6.8 ± 2.9 % ($p = 0.001$) during the first week and further increased to 9.0 ± 3.6 % ($p = 0.004$) after the second week (Fig. 2b). In group C, FMD levels increased from 5.2 ± 3.3 % to 6.9 ± 3.8 % ($p = 0.016$) (Fig. 2c). When the changes in FMD levels during the first week were expressed as the ratio of the post-treatment FMD to the baseline FMD, the aspirin monotherapy, the folic acid monotherapy, and the combination therapy resulted in 1.4 ± 0.4-fold, 1.4 ± 0.4-fold, and 2.9 ± 3.1-fold increases, respectively ($F = 5.63$, $p = 0.0068$). Post-hoc analysis showed that the combination therapy resulted in greater improvement in FMD values than aspirin monotherapy ($p = 0.013$) and folic acid monotherapy ($p = 0.026$), although the latter was not statistically significant after the Bonferroni correction.

For patients in group A, elevation of serum folic acid concentration was observed after the combination therapy and then a decline during the aspirin monotherapy. As expected, serum folic acid concentration for group B patients did not change during aspirin monotherapy but increased as a

result of the combination therapy. For group C patients elevation of serum folic acid concentration was observed after the folic acid monotherapy (Table 2).

Among group C patients, serum Hcy concentration was reduced after the folic acid monotherapy. However, the difference was not significant by ANOVA in serum Hcy levels among baseline, aspirin monotherapy, and the combination of aspirin and folic acid in group A or group B (Table 2). Similarly, there was no significant difference in hsCRP or IL-6 among baseline, aspirin monotherapy, and combination therapy.

Discussion

Endothelial function of patients with type 2 diabetes is seriously degraded compared with that of healthy subjects [20, 21]. Aspirin inhibits platelet aggregation and improves endothelial function, and the effectiveness of low-dose aspirin in the prevention of CVD has been established. Folic acid supplementation reduces Hcy concentration, resulting in improvement of vascular NO bioavailability and overall endothelial function [22]. However, no investigation has been performed to assess the effects of a combination of aspirin and folic acid on vascular endothelial function. The rationale for combining folic acid and aspirin is that:

1. aspirin induces a significant but reversible decrease in serum folic acid concentrations and a small increase in its urinary excretion [15]; and
2. aspirin exerts its anti-inflammatory effects after its conversion into salicylic acid, which has greater antifolic acid activity than the parent compound [16].

Thus, folic acid supplementation in aspirin therapy may be a clinically useful strategy to prevent the impaired balance of this vitamin.

We demonstrated in this study that 1-week combination therapy with low-dose aspirin and folic acid improved FMD of diabetic subjects more efficiently than either drug alone. FMD increased in the aspirin monotherapy group after addition of folic acid, but decreased in the combination therapy group after discontinuation of folic acid. The increases of FMD may be partially attributable to improvement of glycemic control. To minimize the effect of glycemic control improvement during the study period, this study was performed in a cross-over design. Reduction of FMD during the second week in group A clearly showed the beneficial effect of folic acid supplementation of aspirin monotherapy. In this study, folic acid levels were not reduced by aspirin treatment. The absence of effects on serum folic acid levels may be because aspirin was given in a small dose for a short period. Plasma levels of Hcy were significantly reduced by administration of folic acid in group C. It is widely accepted that increased plasma Hcy is associated with increased cardiovascular risk.

However, reduction of plasma Hcy may not be the mechanism by which folic acid supplementation of aspirin therapy further improved endothelial function, because no significant reduction was observed in groups A or B. Another possible mechanism is attenuation of inflammation, because chronic low-grade inflammation may be associated with endothelial dysfunction in diabetes. However, serum levels of hsCRP or IL-6 were not significantly different among baseline, aspirin monotherapy, and combination therapy, and folic acid monotherapy did not reduce hsCRP or IL-6 levels.

This study has several limitations. It was performed with a relatively small number of patients. However, a significant improvement of FMD was demonstrated after the folic acid supplementation. We calculated the sample number adequate for distinguishing the effect of folic acid on FMD with the standard deviations in this cross-over trial by using repeated-measures ANOVA with a Bonferroni correction [23]; a sample size of 32 was verified to be adequate to distinguish differences between this effect. Although the 1-week add-on treatment of folic acid proved beneficial for endothelial function, effects of longer-term administration are not guaranteed. Finally, patients with advanced diabetic complications were excluded because of concern about the effect of anticoagulation therapy, particularly retinal bleeding. These results are, therefore, limited to cases without advanced diabetic complications.

In conclusion, folic acid supplementation of low-dose aspirin therapy resulted in further improvement of endothelial function among patients with type 2 diabetes. On the basis of these results, prospective studies with larger sample sizes for a longer period of time are needed to confirm the clinical relevance of folic acid supplementation of aspirin therapy for the prevention of CVD.

Conflicts of interest No competing financial interests exist.

Human rights statement and informed consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later revision. Informed consent or a substitute for it was obtained from all patients included in the study. If doubt exists whether the research was conducted in accordance with the Helsinki Declaration, the authors must explain the rationale for their approach, and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study. Identifying information of patients of human subjects, including names, initials, addresses, admission dates, hospital numbers, or any other data that might identify patients should not be published in written descriptions, photographs, or pedigrees unless the information is essential for scientific purposes and the patient (or parent guardian) gives written informed consent for publication. If any identifying information about patients is included in the article, the following sentence should also be included: Additional informed consent was obtained

from all patients for which identifying information is included in this article.

References

1. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature. 1993;362:801–9.
2. Nabel EG, Selwyn AP, Ganz P. Large coronary arteries in humans are responsive to changing blood flow: an endotheliumdependent mechanism that fails in patients with atherosclerosis. J Am Coll Cardiol. 1990;16:349–56.
3. Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, et al. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet. 1992;340:1111–5.
4. Boger RH, Ron ES. L-arginine improves vascular function by overcoming deleterious effects of ADMA, a novel cardiovascular risk factor. Altern Med Rev. 2005;10:14–23.
5. Sciacqua A, Grillo N, Quero M, Sesti G, Perticone F. Asymmetric dimethylarginine plasma levels and endothelial function in newly diagnosed type 2 diabetic patients. Int J Mol Sci. 2012;13:13804–15.
6. Razmara M, Hjemdahl P, Ostenson CG, Li N. Platelet hyperprocoagulant activity in type 2 diabetes mellitus: attenuation by glycoprotein IIb/IIIa inhibition. J Thromb Haemost. 2008;6: 2186–92.
7. Colwell JA. Aspirin therapy in diabetes. Diabetes Care. 2004;27(Suppl 1):S72–3.
8. Calvin AD, Aggarwal NR, Murad MH, Shi Q, Elamin MB, Geske JB, et al. Aspirin for the primary prevention of cardiovascular events: a systematic review and meta-analysis comparing patients with and without diabetes. Diabetes Care. 2009;32:2300–6.
9. Williams PC, Coffey MJ, Coles B, Sanchez S, Morrow JD, Cockcroft JR, et al. In vivo aspirin supplementation inhibits nitric oxide consumption by human platelets. Blood. 2005;106: 2737–43.
10. Husain S, Andrews NP, Mulcahy D, Panza JA, Quyyumi AA. Aspirin improves endothelial dysfunction in atherosclerosis. Circulation. 1998;97:716–20.
11. Gao XR, Adhikari CM, Peng LY, Guo XG, Zhai YS, He XY, et al. Efficacy of different doses of aspirin in decreasing blood levels of inflammatory markers in patients with cardiovascular metabolic syndrome. J Pharm Pharmacol. 2009;61:1505–10.
12. Antoniades C, Shirodaria C, Warrick N, Cai S, de Bono J, Lee J, et al. 5-methyltetrahydrofolate rapidly improves endothelial function and decreases superoxide production in human vessels: effects on vascular tetrahydrobiopterin availability and endothelial nitric oxide synthase coupling. Circulation. 2006;114: 1193–201.

13. Verhaar MC, Stroes E, Rabelink TJ. Folates and cardiovascular disease. Arterioscler Thromb Vasc Biol. 2002;22:6–13.
14. Chambers JC, Ueland PM, Obeid OA, Wrigley J, Refsum H, Kooner JS. Improved vascular endothelial function after oral B vitamins: an effect mediated through reduced concentrations of free plasma homocysteine. Circulation. 2000;102:2479–83.
15. Lawrence VA, Loewenstein JE, Eichner ER. Aspirin and folatebinding: in vivo and in vitro studies of serum binding and urinary excretion of endogenous folate. J Lab Clin Med. 1984;103:944–8.
16. Baggott JE, Morgan SL, Ha T, Vaughn WH, Hine RJ. Inhibition of folate-dependent enzymes by non-steroidal anti-inflammatory drugs. Biochem J. 1992;282(Pt 1):197–202.
17. Das UN. Folic acid says NO to vascular diseases. Nutrition. 2003;19:686–92.
18. Kawano N, Emoto M, Mori K, Yamazaki Y, Urata H, Tsuchikura S, et al. Association of endothelial and vascular smooth muscle dysfunction with cardiovascular risk factors, vascular complications, and subclinical carotid atherosclerosis in type 2 diabetic patients. J Atheroscler Thromb. 2012;19:276–84.
19. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, et al. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the international brachial artery reactivity task force. J Am Coll Cardiol. 2002;39:257–65.
20. Nair BM, Viswanathan V, Snehalatha C, Mohan RS, Ramachandran A. Flow mediated dilatation and carotid intimal media thickness in South Indian type 2 diabetic subjects. Diabetes Res Clin Pract. 2004;65:13–9.
21. Tian J, Wen Y, Yan L, Cheng H, Yang H, Wang J, et al. Vascular endothelial dysfunction in patients with newly diagnosed type 2 diabetes and effects of 2 and 5-year multifactorial intervention. Echocardiography. 2011;28:1133–40.
22. Antoniades C, Antonopoulos AS, Tousoulis D, Marinou K, Stefanadis C. Homocysteine and coronary atherosclerosis: from folate fortification to the recent clinical trials. Eur Heart J. 2009;30:6–15.
23. Julious SA. Sample sizes for clinical trials with normal data. Stat Med. 2004;23:1921–86.

Figure legends

Fig. 1 The study protocol. Arrows indicate the times of FMD measurements and blood sampling.

Fig. 2 Changes in FMD during the 2-week study period for patients of group A (a) and group B (b), and changes in FMD during the 1-week study period for patients of group C (c). Data are presented

as mean ± SD.

Table 1. Clinical characteristics at baseline

	All subjects	group A	group B	group C	P-value
Number of patients	46	15	17	14	—
Gender (male/female)	20/26	7/8	5/12	8/6	0.29
Habitual drinking (+/-)	15/31	5/10	3/14	7/7	0.17
Age (years)	57±14	56±14	55±15	60±13	0.63
BMI (kg/m^2)	26.5±6.0	28.2±8.1	26.6±5.3	24.5±3.8	0.27
Duration of diabetes (years)	8.9±8.2	7.6±7.7	9.9±2.4	7.6±6.2	0.41
Systolic blood pressure (mmHg)	125±13	129±13	124±11	121±16	0.31
Diastolic blood pressure (mmHg)	75±11	76±10	76±12	74±13	0.91
RAS blockade (ACEI or ARB) (+/-)	6/40	2/13	1/16	3/11	0.46
Statin (+/-)	13/33	3/12	4/13	6/8	0.35
Fasting plasma glucose (mg/dL)	153±46	155±65	159±38	145±27	0.69
HbA1c (%)	9.3±1.6	9.1±1.5	9.3±1.6	9.5±1.8	0.81
LDL-C (mg/dL)	128±37	136±34	130±40	116±36	0.37
HDL-C (mg/dL)	52±13	54±20	50±8	52±10	0.68
TG (mg/dL)	146±61	154±77	141±54	142±54	0.81
Creatinine (mg/dL)	0.68±0.26	0.71±0.41	0.66±0.15	0.68±0.14	0.82
Folic acid (ng/mL)	8.1±3.5	7.6±4.0	7.8±2.8	9.1±3.7	0.49
Homocysteine (μmol/L)	9.1±3.1	8.7±3.3	8.8±1.7	9.8±4.2	0.55
hsCRP (mg/L)	1.63±2.24	1.86±1.82	1.51±1.97	1.53±3.0	0.9
IL-6 (pg/mL)	3.3±3.8	2.7±1.8	4.3±5.7	2.7±2.0	0.39
FMD (%)	4.7±2.6	3.8±2.1	5.1±2.2	5.2±3.4	0.25

RAS: Renin-angiotensin system ACEI: angiotensin converting enzyme inhibitor, ARB: angiotensin receptor blocker,
hsCRP: high-sensitivity C-reactive protein
Data are presented as means±S.D. or numbers of patients.
Most parameters were measured at hospitalization.
One way ANOVA or Chi-square test was used for the comparison among the groups..

Table 2. Changes of clinical parameters

	group A (n=15)			group B (n=17)			group C (n=14)	
	Baseline	*Aspirin plus folic acid*	*Aspirin*	*Baseline*	*Aspirin*	*Aspirin plus folic acid*	*Baseline*	*Folic acid*
Folic acid (ng/mL)	7.6±3.8	118.8±101.0 ** #	23.6±5.4 **	7.8±2.7	13.6±18.4	204±162.9 ** #	8.7±4.1	200.0±166.3 **
Hcy (μmol/l)	8.7±3.2	6.7±1.6	6.9±1.7	8.8±1.6	8.9±2.0	7.9±1.7	9.9±4.2	8.6±2.9 *
hsCRP (mg/L)	1.86±1.82	1.16±1.3	0.89±0.91	1.51±1.97	0.71±0.52	0.55±0.44	1.53±3.0	1.50±2.36
IL-6 (pg/mL)	2.7±1.7	2.2±1.2	2.0±0.9	4.3±5.5	3.5±4.5	2.8±3.3	2.7±2.0	2.6±2.4

Descriptions for each abbreviation are given in text.
Data are presented as means±SD.
No significant difference was obtained in Hcy, hsCRP, or IL-6 among baseline, aspirin, and aspirin plus folic acid by ANOVA.
*P<0.05, **P<0.001 vs. values at baseline. #P<0.001 vs. values after aspirin mono-therapy.

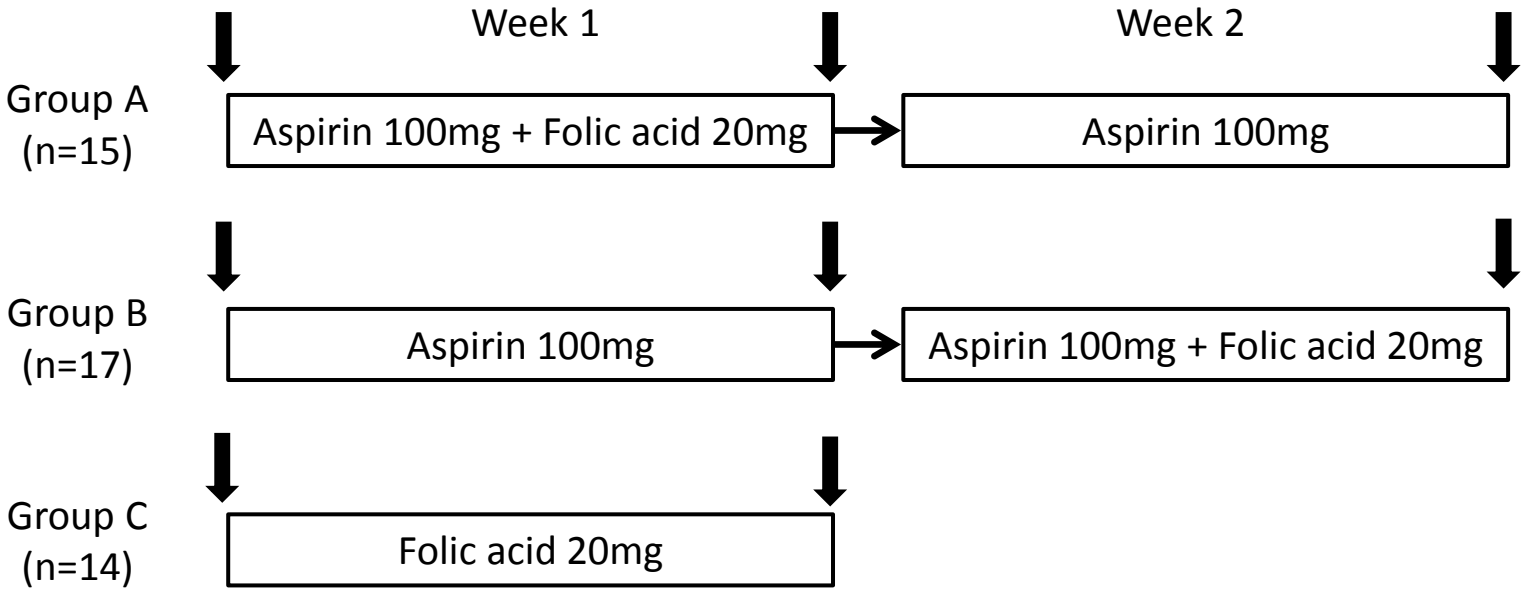

Figure 1. Sato, et al.

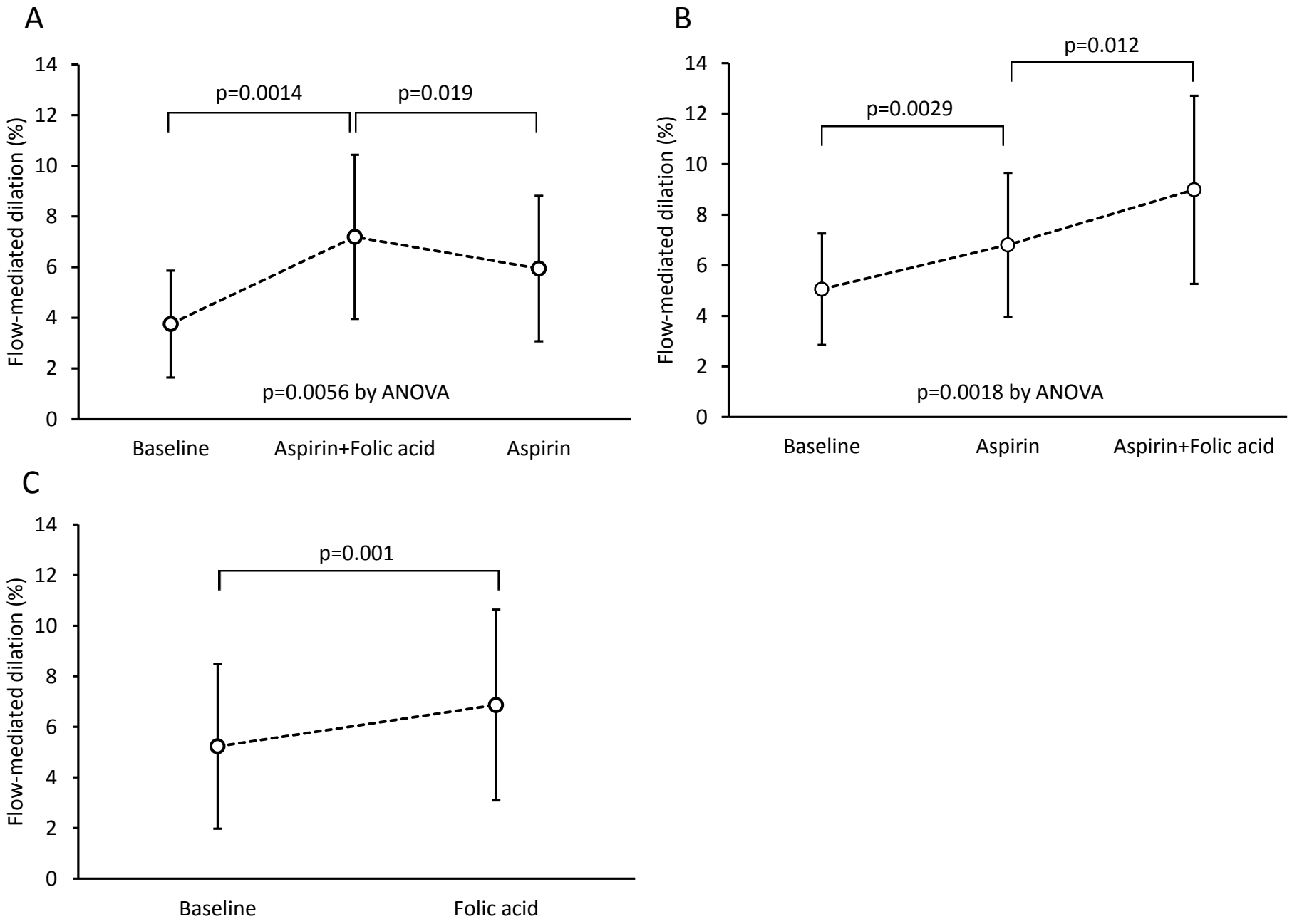

Figure 2. Sato, et al.