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FULL PAPER

Pharmacology

Effects of leucovorin (folinic acid) in the methotrexate-treated rat brain

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ABSTRACT. Folinic acid (FA) is generally administered to patients with CNS tumors in order to treat severe neurological disorders caused by methotrexate (MTX); therefore, we herein examined the effects of the co-administration of FA on MTX concentrations in the rat brain and cerebrospinal fluid (CSF) as well as the pharmacokinetics of MTX. MTX was intravenously or intrathecally administered to rats with or without FA. MTX concentrations were assessed by HPLC. No significant differences were observed in pharmacokinetic parameters, including k_{el}, V_d, AUC, Cl_{tot} and $t_{1/2}$, between the FA-treated and non-treated groups. MTX concentrations were not significantly different in the brain or CSF 6 hr after the intrathecal administration of MTX. However, compare to intravenous administration of MTX, intravenous administration of both FA and MTX significantly decreased MTX concentrations in the brains and CSF. These results suggest that FA inhibits the influx of MTX into the brain and CSF, possibly by competing with folate carriers, but has no effect on its efflux from these regions. Therefore, FA may be administered to CNS tumor patients receiving intrathecal MTX therapy in order to treat the adverse effects of MTX without affecting its concentrations in the brain and CSF.

KEY WORDS: blood-brain barrier, blood-CSF barrier, folate carrier, folinic acid, methotrexate

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Methotrexate (MTX) is an anticancer drug that is used to treat central nervous system (CNS) tumors. However, its influx into the CNS is strongly restricted because of its water solubility. The CNS is also protected from xenobiotics such as MTX by the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (B-CSFB) at the choroid plexus [5, 14, 17]. Various methods have been developed to deliver MTX into the CNS at sufficient concentrations, such as its administration via an intravenous (*i.v.*) route at high doses and an intrathecal (*i.t.*) route in humans and animals [1, 3, 4, 6, 8, 9, 13].

Since MTX is a folate analog, its carrier-mediated transportation generally depends on folate carriers, such as reduced folate carriers (RFC), proton-coupled folate transporters (PCFT), and folate receptor-mediated endocytosis (FR). These carriers are also expressed at the BBB and B-CSFB. RFC is expressed at the apical membrane. RFC is a bidirectional transporter and since it is an organic anion antiporter driven by the organic phosphate gradient, it favors transport from CSF into the ependymal cells. In adult humans, PCFT, which transport folates from blood into the cerebrospinal fluid (CSF), are expressed along the basolateral membrane of the choroid plexus, and FR, extracting folate from CSF, are abundantly expressed at the apical brush-border membrane within the CSF and, to a lesser extent, at the basolateral membrane [7, 10, 18, 20, 21].

However, multidrug transporters of the ATP-binding cassette family, such as P-glycoprotein (P-gp), and multidrug-resistant proteins (MRPs) may also play an important role in regulating the distribution and efflux of MTX in the brain and choroid plexus. We previously reported that the potent P-gp and MRP1 modulator, cyclosporin A (CysA) and specific P-gp modulator, rhodamine 123 (Rho123) potentiated MTX concentrations in the rat brain when MTX was administered *i.t.* However, neither modulator potentiated MTX concentrations when it was administered *i.v.* [11, 12]. Based on these findings, P-gp appears to be crucially involved in the efflux of MTX from the rat brain. However, it currently remains unclear whether folate carriers contribute to the efflux of MTX from the brain and CSF.

Folinic acid (FA) supplementation is essential in patients with CNS tumors for the treatment of severe neurological disorders caused by MTX [2]. It may disturb the distribution of MTX in the brain because it is a folate derivative and, thus, may share folate carriers with MTX.

A previous study reported that a folate deficiency disrupted BBB function by targeting P-gp and tight junctions and also that FA supplementation restored BBB function to normal levels [18]. MRP have been shown to transport not only MTX, but also folate derivatives, such as folic acid and FA [16, 19]. These findings suggest that FA affects the retention of MTX in the brain

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when used to treat MTX-related adverse effects. Therefore, we herein investigated the effects of co-medication with FA and *i.v.*- or *i.t.*-administered MTX on the retention of MTX in the rat brain.

MATERIALS AND METHODS

Animals

Since male rats were generally larger than females, we thought male rats more suitable to administer drugs intrathecally. Thus, male Sprague-Dawley rats (8 weeks old) were obtained from CLEA Japan Inc. (Tokyo, Japan) and utilized in all experiments the next week (weighing between 251 and 310 g). Animals were maintained under a 12:12 hr light-dark cycle and had free access to food and water prior to experiments. The experiments performed in the present study were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and approved by the Committee of Animal Experiments, Tokyo University of Agriculture and Technology.

Chemicals

MTX and its polyglutamates were purchased from Schricks Laboratories (Jona, Switzerland). MTX solution was prepared at 20 mg/m*l* by diluting a commercially available injectable formulation (Methotrexate® Injection, Takeda Pharmaceutical Co., Ltd., Osaka, Japan) with sterilized saline. FA solution (Leucovorin®) was purchased as a hydrochloride salt (Pfizer Inc., New York, NY, U.S.A.).

Drug administration and sampling protocol

We considered two points to determine the FA and MTX dose. Primarily, in order to avoid increases in intracranial pressure, *i.t.* injections should be performed after removing the same volume of CSF as the injection volume. Since we could collect about 0.2 m*l* CSF from the rats, we determined the FA and MTX injection volume as 0.1 m*l* respectively.

Secondly, we determined MTX and FA dose as following. When MTX is administered intrathecally, the clinical dose of MTX is 0.2–0.4 mg/kg in human. When MTX is administered intravenously, it is 5–10 mg/body in human. However, since some of MTX is stored as polyglutamates, we needed to analyze the polyglutamate concentrations. In addition, MTX polyglutamates' peaks were less than monoglutamate MTX. For that reason, we administered MTX to rats at 2 mg/body (0.1 m*l*) to detect MTX and its polyglutamates in rat brains and CSF.

Next, FA is generally administered at 0.1–0.25 mg/kg to prevent the adverse effects by MTX in human. However, since one of our aims in this study was to examine if FA show competitive inhibition to MTX, we defined FA dose as the maximum dose we could administer intrathecally (0.3 mg/0.1 m*l*).

The administration of all drugs was conducted under anesthesia with pentobarbital (50 mg/kg, intraperitoneally). In order to minimalize leaking CSF from the halls by the needles, MTX (2 mg/body) and FA (0.3 mg/body) were simultaneously injected into animals via an *i.v.* or *i.t.* route.

We defined 5 groups as follows: group Miv: MTX (*i.v.*) + saline (*i.t.*), group Mit: MTX (*i.t.*), group Miv+Fiv: MTX (*i.v.*) + FA (*i.v.*) + saline (*i.t.*), group Mit+Fiv: MTX (*i.t.*) + FA (*i.v.*), and group Mit+Fit: MTX (*i.t.*) + FA (*i.t.*) (Table 1).

Blood (0.2 m*l*) was sampled from the caudal vein 1, 2, 3, 4, 5 and 6 hr after drug administration. In our preliminary study, the half-lives of *i.v.*- and *i.t.*-administered MTX were 35 ± 3.2 and 84 ± 20 min, respectively.

Rats in each group were euthanized 6 hr after drug administration following the sampling of CSF (0.1 m*l*) from the cisterna, and brains were collected (n=5, respectively). In order to prevent brains from contamination with MTX in blood for as long as possible, brains were isolated after removing all blood from the body. Isolated brains were sagitally divided into two pieces at the median line for the analysis of MTX concentrations and stored at −80°C until used.

Sample preparation

One piece of the brain was homogenized with methanol (50 m*l*) to extract MTX and MTX polyglutamates. The homogenate was centrifuged at 3,000 × *g* for 20 min in order to separate the clear liquid layer and residue. The clear layer was evaporated, and the residue was mixed with 10 mM sodium acetate buffer (pH 1.6, 50 m*l*) in order to dissolve the water-soluble polyglutamates of MTX. The mixture was then centrifuged at 3,000 × *g* for 20 min. The clear liquid layer obtained was mixed with the layer that was evaporated to dryness.

In order to purify and concentrate MTX and its polyglutamates, a mixture of 20 m*l* was subjected to solid phase extraction (Sep-Pak® Plus C18 cartridge, Waters Corp., Milford, MA, U.S.A.). MTX and its polyglutamates were eluted with 2 m*l* of 50% methanol solution (pH 7.0), and the eluate was then subjected to a HPLC analysis for MTX.

Plasma and CSF samples (0.1 m*l*) were added to $HClO_4$ (0.2 m*l*) to remove plasma proteins, and were then centrifuged at 12,000 × *g* for 2 min to divide the clear liquid layer and residue. The clear liquid layer was subjected to a HPLC analysis for MTX.

Table 1. Definition of administration groups

Group	MTX	FA
Miv	i.v.	
Mit	i.t.	
Miv+Fiv	i.v.	i.v.
Mit+Fiv	i.t.	i.v.
Mit+Fit	i.t.	i.t.

Miv: MTX (i.v.) + saline (i.t.), Mit: MTX (i.t.), Miv+Fiv: MTX (i.v.) + FA (i.v.) + saline (i.t.), Mit+Fiv: MTX (i.t.)+FA (i.v.), Mit+Fit: MTX (i.t.)+FA (i.t.). Each value is the mean ± S.D. (n=5). The doses of MTX and FA administered were 2 and 0.3 mg/body, respectively.

HPLC analysis

MTX and its polyglutamates were analyzed using a HPLC system. The mobile phase consisted of 10 mM acetate buffer (pH 1.6) and acetonitrile (90:10, v/v), and the effluent was monitored by a UV detector (SPD-6A®, Shimadzu, Kyoto, Japan) at a wavelength of 313 nm. The analytical column was an ion-exchange column (PARTICIL® 10 SCX, 4.6 × 250 mm, Whatman, part of GE Healthcare, Tokyo, Japan). Plasma samples were monitored for MTX (monoglutamate) only because MTX polyglutamates are typically found in red blood cells. MTX and its polyglutamates were measured in CSF and brain samples. The sum of the concentrations of MTX and its polyglutamates was expressed as total MTX. However, MTX pentaglutamate (-glu5) and hexaglutamate (MTX-glu6) were not analyzed in brain samples because negligible amounts were obtained in a preliminary study. The recoveries of MTX monoglutamate, diglutamate, triglutamate, and tetraglutamate were 93.2 ± 4.1, 79.2 ± 5.5, 72.7 ± 4.0 and 70.2 ± 1.7%, respectively (n=5), at 1 μg/m*l*. Their coefficients of variation (CV) were 4.4, 6.9, 5.5 and 2.5%, respectively. Interday CV values in the assay ranged between 1.6 and 5.8% with a limit of quantification of 3.5 *n*g/m*l* at a signal-to-noise ratio of 3 (n=5).

Pharmacokinetic analysis

A one-compartment open model was used to analyze the pharmacokinetics of MTX. The plasma concentration at time 0 (C_0) and elimination rate constant (k_{el}) in the following equation were calculated using a non-linear least-squares fitting.

$$CP=C_0\ e^{-kel\ t}$$

where Cp and t represent the plasma concentration and time after the administration of MTX, respectively.

The area under the plasma concentration-time curve (AUC) was obtained as a sum of the area from 0 to the last sampling time by the trapezoidal method. The elimination half-life ($t_{1/2}$), apparent volume of distribution (V_d), and total body clearance (Cl_{tot}) were calculated using the following equations:

$$t_{1/2}=0.693/k_{el}$$
$$V_d=Dose/C_0$$
$$Cl_{tot}=Dose/AUC$$

Statistical analysis

Data are displayed as means± SD. Differences in mean values between groups were analyzed by Scheffé's multiple comparison test after a one-way ANOVA. Equal variances among the groups were confirmed by Bartlett's test. Since we examined if FA competitively inhibit MTX to penetrate into the brains and CSF, comparisons between Miv and Miv+Fiv were conducted using the one-sided Student's *t*-test. Differences were considered to be significant at $P<0.05$.

RESULTS

As shown in Fig. 1, no significant differences were observed in plasma concentration-time courses between the FA-treated and non-treated groups. Furthermore, pharmacokinetic parameters, including k_{el}, V_d, AUC, Cl_{tot} and $t_{1/2}$, were not significantly different between the FA-treated and non-treated groups (Table 2).

Figure 2A shows MTX concentrations in the brain and CSF 6 hr after the administration of MTX. No significant differences were observed in MTX concentrations in the brain or CSF among the *i.t.*-administered groups: Mit, Mit+Fiv and Mit+Fit, suggesting that FA did not significantly affect MTX concentrations in the brain or CSF regardless of its *i.v.* or *i.t.* administration.

In contrast, as shown in Fig. 2B, MTX concentrations in the brain and CSF were lower in the Miv+Fiv group than in the Miv group. MTX concentrations in the brain and CSF were significantly lower in the Miv+Fiv group than in the Miv group ($P<0.05$).

The brain to CSF MTX concentration ratio was compared among these groups, as shown in Fig. 3. The *i.t.*-administered groups: Mit+Fiv and Mit+Fit, had a slightly higher ratio than the Mit group. No significant differences were observed between the Miv and Miv+Fiv groups, suggesting that MTX concentrations were not shifted to the brains or the CSF.

DISCUSSION

Since FA supplementation is essential for patients with CNS tumors to treat severe neurological disorders caused by MTX [2], the mechanisms by which it affects MTX in the brain and CSF need to be elucidated in more detail. Therefore, there were two aims in the present study.

(1) To clarify whether folate carriers (RFC, PCFT and FR) are involved in efflux of MTX from the CNS.

(2) To clarify whether folate carriers are involved in the influx of MTX into the CNS from blood.

Figure 1 and Table 2 showed that FA did not have a significant pharmacokinetic interaction with MTX (2 mg/body) at the dose administered (0.3 mg/body), suggesting that FA did not affect the renal excretion of MTX.

FA did not significantly affect MTX concentrations in the brain or CSF when MTX was administered *i.t.*, as shown in Fig. 2A. MTX concentrations in the CSF were not significantly different between the Mit+Fiv and Mit+Fit groups, indicating that FA did not affect MTX transporters on the basolateral membrane or apical brush-border membrane at the B-CSFB.

Therefore, folate transporters do not appear to contribute to the efflux of MTX from the brain and CSF.

Furthermore, as shown in Fig. 2B, FA inhibited the influx of MTX into the brain and CSF. A significant difference was

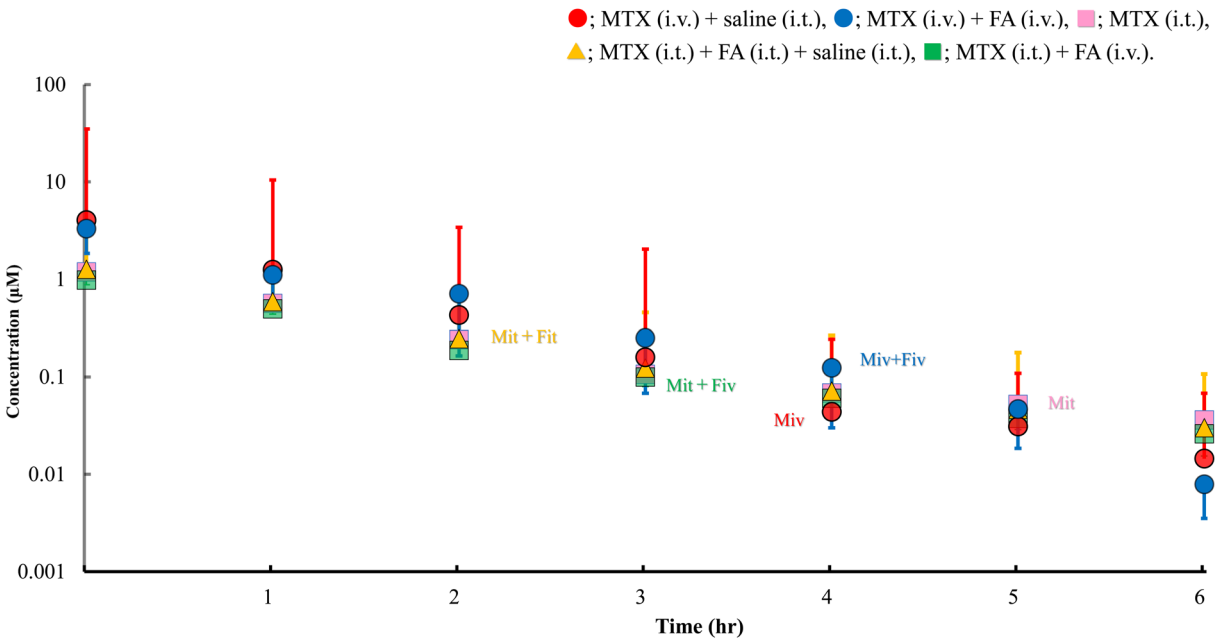

Fig. 1. MTX concentrations in plasma after the administration of MTX with or without folinic acid to rats. Each value is the mean ± S.D. (n=5). The doses of MTX and FA administered were 2 and 0.3 mg/body, respectively. No significant differences were observed in pharmacokinetic parameters, including k_{el}, V_d, AUC, CL_{tot} and $t_{1/2}$, between the FA-treated and non-treated groups.

Table 2. Pharmacokinetic parameters of MTX after administrations of MTX with or without FA to rats

Group	C_0 (μM)	k_{el} (*l*/hr)	AUC (μM•hr)	V_d (*l*/body)	Cl_{tot} (*l*/hr)	$t_{1/2}$ (min)
Mit	1.19 ± 0.27	0.72 ± 0.12	1.71 ± 0.15	4.59 ± 1.03	10.9 ± 1.0	64 ± 10
Mit+Fiv	0.98 ± 0.10	0.76 ± 0.07	1.42 ± 0.15	4.71 ± 0.52	13.4 ± 1.8	57 ± 6.6
Mit+Fit	1.26 ± 0.25	0.77 ± 0.14	1.77 ± 0.13	3.91 ± 0.56	10.5 ± 0.7	60 ± 8.0
Miv	4.04 ± 1.81	1.23 ± 0.12	3.95 ± 1.79	2.67 ± 1.05	12.2 ± 4.2	35 ± 3.4
Miv+Fiv	3.31 ± 1.46	1.16 ± 0.15	3.91 ± 2.11	2.30 ± 0.70	10.5 ± 3.2	38 ± 4.2

C_0; initial concentration, k_{el}; elimination rate constant, AUC; area under the plasma concentration-time curves, V_d; volume of distribution, Cl_{tot}; total body clearance, $t_{1/2}$; elimination half-life, Miv: MTX (i.v.)+ saline (i.t.), Mit: MTX (i.t.), Miv+Fiv: MTX (i.v.) +FA (i.v.)+ saline (i.t.), Mit+Fiv: MTX (i.t.) +FA (i.v.), Mit+Fit: MTX (i.t.)+FA (i.t.). Each value is a mean ± S.D. (n=5). Doses of MTX and FA were 2 and 0.3 mg/body, respectively. Each parameter was not significantly different among these groups.

observed in MTX concentrations in the brain and CSF between the Miv and Miv+Fiv groups. In addition, the brain to CSF MTX concentration ratio was not significantly different between the Miv and Miv+Fiv groups (Fig. 3), indicating that FA decreased MTX concentrations without shifting to the brains or the CSF. This indicates that FA did not only inhibit the influx of MTX into the CSF.

In other words, it is suggested that FA impaired both the BBB and B-CSFB functions in charge of PCFT and a part of FR to penetrate MTX into the brains and CSF [18, 20, 21]. Probably, although FA competitively impaired all of the folate carriers, the physiological function to influx folates from blood into the CNS is superior to the function to efflux folates from the CNS, because FA did not significantly impaired to efflux intrathecally administered MTX in this study.

Hence, it is suggested that FA competitively inhibit to influx MTX into the brain and CSF without significant pharmacokinetic interaction.

In conclusion, the results of the present study suggest that folate carriers play an important role in the influx of MTX into the brain and CSF, but not from its efflux from the CNS.

Regarding the optimal dosage schedule, MTX is classified one of S-phase specific but self-limiting drugs. While the optimal schedule for the S-phase specific drugs was one which provided effective serum concentrations at intervals shorter than the median DNA synthesis time of cancers, increasing the concentration of S-phase specific but self-limiting drugs above some minimum level does not increase the rate of cancer cells but does increase host toxicity [15]. Hence, it is important for a successful chemotherapy to keep MTX at minimum level for a longer time.

In addition, though FA did not affect plasma MTX concentration in this study, it is known that FA supplement is clinically available to decrease systemic host toxicity.

Therefore, even though FA were administered, we might not have to change the dosage schedule. Thus, FA may be administered

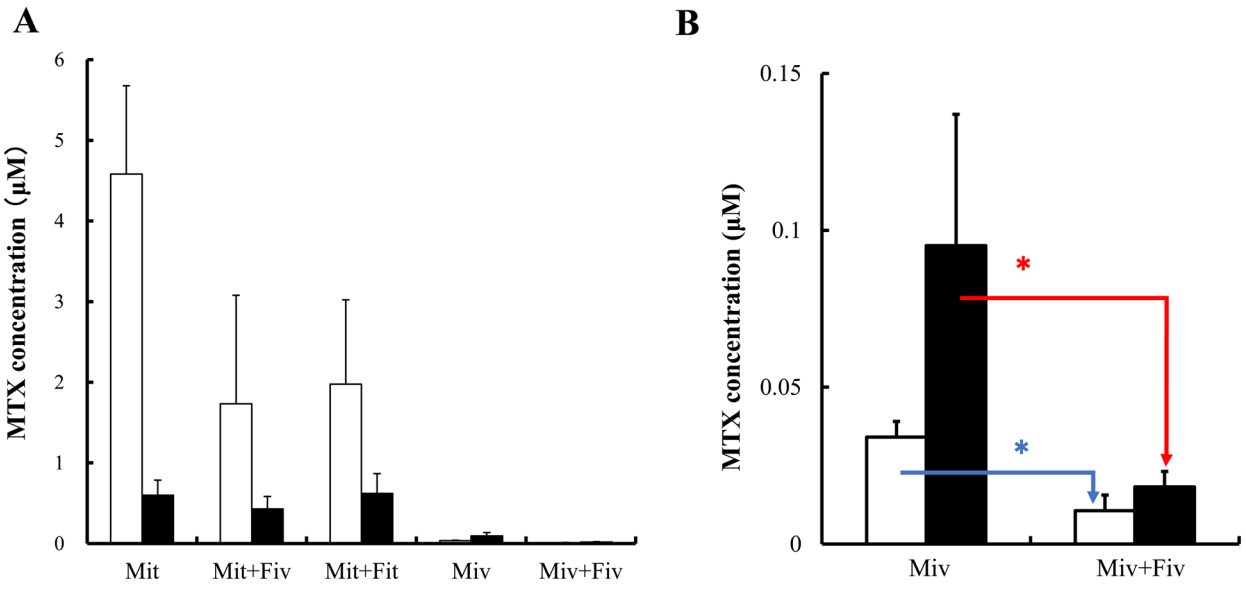

Fig. 2. Total MTX concentrations in the brain and CSF after the administration of MTX with or without FA to rats. This figure comprises charts A and B. Chart A shows MTX concentrations in the brain and CSF of all groups. Chart B shows MTX concentrations in the brain and CSF of intravenously MTX-administered groups (Miv, Miv+Fiv). The following abbreviations are used: Miv: MTX (*i.v.*) + saline (*i.t.*), Mit: MTX (*i.t.*), Miv+Fiv: MTX (*i.v.*) + FA (*i.v.*) + saline (*i.t.*), Mit+Fiv: MTX (*i.t.*) + FA (*i.v.*), Mit+Fit: MTX (*i.t.*) + FA (*i.t.*). White and black bars represent CSF and brain concentrations, respectively. Each value is the mean ± S.D. (n=5). The doses of MTX and FA administered were 2 and 0.3 mg/body, respectively. *Between Miv+Fiv and Miv, statistical difference was seen in brain, as well as CSF (P<0.05).

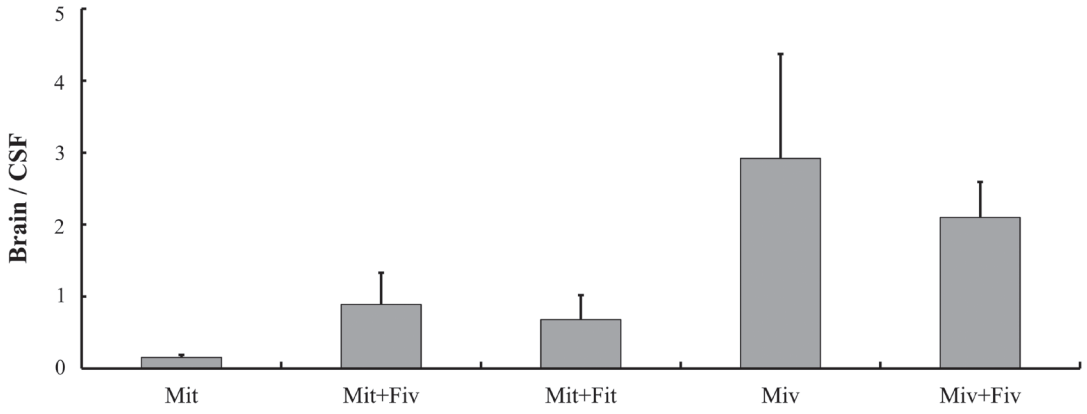

Fig. 3. The brain and CSF MTX concentration ratio 6 hr after the administration of MTX with or without FA to rats. Miv group, MTX (*i.v.*)+saline (*i.t.*); Mit, MTX (*i.t.*); Miv+Fiv group, MTX (*i.v.*)+FA (*i.v.*)+saline (*i.t.*); Mit+Fiv group, MTX (*i.t.*)+FA (*i.v.*); and Mit+Fit group, MTX (*i.t.*)+FA (*i.t.*). MTX and FA are abbreviations for methotrexate and folinic acid, respectively. Each value is presented as the mean± SD (n=5). The doses of MTX and FA were 2 and 0.3 mg/body, respectively. There was no significant difference among the *i.t.*-administered MTX groups (P<0.05, Scheffé's multiple comparison test).

to patients with CNS tumors receiving *i.t.* MTX therapy to treat the adverse effects of MTX without affecting its concentrations in the brain and CSF; however, FA may decrease these concentrations in patients receiving *i.v.* MTX therapy at a high dose.

REFERENCES

1. Abelson, H. T., Kufe, D. W., Skarin, A. T., Major, P., Ensminger, W., Beardsley, G. P. and Canellos, G. P. 1981. Treatment of central nervous system tumors with methotrexate. *Cancer Treat. Rep.* **65** Suppl 1: 137–140. [Medline]
2. Bhojwani, D., Sabin, N. D., Pei, D., Yang, J. J., Khan, R. B., Panetta, J. C., Krull, K. R., Inaba, H., Rubnitz, J. E., Metzger, M. L., Howard, S. C., Ribeiro, R. C., Cheng, C., Reddick, W. E., Jeha, S., Sandlund, J. T., Evans, W. E., Pui, C. H. and Relling, M. V. 2014. Methotrexate-induced neurotoxicity and leukoencephalopathy in childhood acute lymphoblastic leukemia. *J. Clin. Oncol.* **32**: 949–959. [Medline] [CrossRef]
3. Bleyer, W. A. and Poplack, D. G. 1985. Prophylaxis and treatment of leukemia in the central nervous system and other sanctuaries. *Semin. Oncol.*

12: 131–148. [Medline]
4. Genoni, S., Palus, V., Eminaga, S. and Cherubini, G. B. 2016. Safety of intrathecal administration of cytosine arabinoside and methotrexate in dogs and cats. *Vet. Comp. Oncol.* **14**: 331–336. [Medline] [CrossRef]
5. Ghersi-Egea, J. F. and Strazielle, N. 2001. Brain drug delivery, drug metabolism, and multidrug resistance at the choroid plexus. *Microsc. Res. Tech.* **52**: 83–88. [Medline] [CrossRef]
6. Glantz, M. J., Cole, B. F., Recht, L., Akerley, W., Mills, P., Saris, S., Hochberg, F., Calabresi, P. and Egorin, M. J. 1998. High-dose intravenous methotrexate for patients with nonleukemic leptomeningeal cancer: is intrathecal chemotherapy necessary? *J. Clin. Oncol.* **16**: 1561–1567. [Medline] [CrossRef]
7. Halwachs, S., Lakoma, C., Schäfer, I., Seibel, P. and Honscha, W. 2011. The antiepileptic drugs phenobarbital and carbamazepine reduce transport of methotrexate in rat choroid plexus by down-regulation of the reduced folate carrier. *Mol. Pharmacol.* **80**: 621–629. [Medline] [CrossRef]
8. Kaleita, T. A., Reaman, G. H., MacLean, W. E., Sather, H. N. and Whitt, J. K. 1999. Neurodevelopmental outcome of infants with acute lymphoblastic leukemia: a Children's Cancer Group report. *Cancer* **85**: 1859–1865. [Medline] [CrossRef]
9. Kamps, W. A., Bökkerink, J. P., Hählen, K., Hermans, J., Riehm, H., Gadner, H., Schrappe, M., Slater, R., van den Berg-de Ruiter, E., Smets, L. A., de Vaan, G. A., Weening, R. S., van Weerden, J. F., van Wering, E. R. and den der Does-van den Berg, A. 1999. Intensive treatment of children with acute lymphoblastic leukemia according to ALL-BFM-86 without cranial radiotherapy: results of Dutch Childhood Leukemia Study Group Protocol ALL-7 (1988–1991). *Blood* **94**: 1226–1236. [Medline]
10. Matherly, L. H. and Hou, Z. 2008. Structure and function of the reduced folate carrier a paradigm of a major facilitator superfamily mammalian nutrient transporter. *Vitam. Horm.* **79**: 145–184. [Medline] [CrossRef]
11. Ogushi, N., Sasaki, K. and Shimoda, M. 2015. Effect of cyclosporin on distribution of methotrexate into the brain of rats. *J. Vet. Med. Sci.* **77**: 1171–1173. [Medline] [CrossRef]
12. Ogushi, N., Sasaki, K. and Shimoda, M. 2017. CAN a P-gp modulator assist in the control of methotrexate concentrations in the rat brain? -inhibitory effects of rhodamine 123, a specific substrate for P-gp, on methotrexate excretion from the rat brain and its optimal route of administration. *J. Vet. Med. Sci.* **79**: 320–327. [Medline] [CrossRef]
13. Reiter, A., Schrappe, M., Tiemann, M., Ludwig, W. D., Yakisan, E., Zimmermann, M., Mann, G., Chott, A., Ebell, W., Klingebiel, T., Graf, N., Kremens, B., Müller-Weihrich, S., Plüss, H. J., Zintl, F., Henze, G. and Riehm, H. 1999. Improved treatment results in childhood B-cell neoplasms with tailored intensification of therapy: A report of the Berlin-Frankfurt-Münster Group Trial NHL-BFM 90. *Blood* **94**: 3294–3306. [Medline]
14. Serlin, Y., Shelef, I., Knyazer, B. and Friedman, A. 2015. Anatomy and physiology of the blood-brain barrier. *Semin. Cell Dev. Biol.* **38**: 2–6. [Medline] [CrossRef]
15. Skipper, H. E., Schabel, F. M. Jr., Mellett, L. B., Montgomery, J. A., Wilkoff, L. J., Lloyd, H. H. and Brockman, R. W. 1970. Implications of biochemical, cytokinetic, pharmacologic, and toxicologic relationships in the design of optimal therapeutic schedules. *Cancer Chemother. Rep.* **54**: 431–450. [Medline]
16. Soontornmalai, A., Vlaming, M. L. and Fritschy, J. M. 2006. Differential, strain-specific cellular and subcellular distribution of multidrug transporters in murine choroid plexus and blood-brain barrier. *Neuroscience* **138**: 159–169. [Medline] [CrossRef]
17. Wolburg, H. and Paulus, W. 2010. Choroid plexus: biology and pathology. *Acta Neuropathol.* **119**: 75–88. [Medline] [CrossRef]
18. Wang, X., Cabrera, R. M., Li, Y., Miller, D. S. and Finnell, R. H. 2013. Functional regulation of P-glycoprotein at the blood-brain barrier in proton-coupled folate transporter (PCFT) mutant mice. *FASEB J.* **27**: 1167–1175. [Medline] [CrossRef]
19. Zeng, H., Chen, Z. S., Belinsky, M. G., Rea, P. A. and Kruh, G. D. 2001. Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: effect of polyglutamylation on MTX transport. *Cancer Res.* **61**: 7225–7232. [Medline]
20. Zhao, R., Diop-Bove, N., Visentin, M. and Goldman, I. D. 2011. Mechanisms of membrane transport of folates into cells and across epithelia. *Annu. Rev. Nutr.* **31**: 177–201. [Medline] [CrossRef]
21. Zhao, R. and Goldman, I. D. 2013. The proton-coupled folate transporter: physiological and pharmacological roles. *Curr. Opin. Pharmacol.* **13**: 875–880. [Medline] [CrossRef]