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Profiles of methotrexate in blood and CSF following intranasal and intravenous administration to rats

Feng Wang, Xinguo Jiang*, Wei Lu

Department of Pharmaceutics, School of Pharmacy, Fudan University, Shanghai 200032, PR China

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Abstract

The aim of this paper was to investigate the levels of methotrexate (MTX) in blood and the cerebrospinal fluid (CSF) in rats to find out whether there is any direct drug transport from nasal cavity to CSF following intranasal administration. Methotrexate was administered to male Sprague–Dawley rats either intranasally or intravenously. Drug concentrations were determined from CSF and plasma samples collected from the cisterna magna and caudal vein, respectively. To collect CSF sample continuously, blank artificial CSF was infused into the lateral ventricle. The plasma levels achieved following intranasal administration were significantly lower than those after intravenous administration ($P < 0.01$) were, while CSF concentrations achieved after intranasal administration were significantly higher than those after intravenous administration ($P < 0.01$). The ratio of the AUC_{CSF} value between the intranasal route and the intravenous injection was 13.76, whereas the absolute bioavailability was only 6.3%, the drug targeting index (DTI) of nasal route was 21.7. In conclusion, these results showed that the antineoplastic MTX must be directly transported from the nasal cavity into the CSF in rats.

Keywords: Cerebrospinal fluid (CSF); Methotrexate; Intranasal administration; Intravenous administration

1. Introduction

It is well known that the blood–brain barrier (BBB) restricts the transport of most substances from the systemic circulation to the central nervous system (CNS), which is surrounded by cerebrospinal fluid (CSF), in order to maintain the stable environment. However, this barrier is also the primary obstacle for the delivery of therapeutic substances to the brain. There have been many researches attempting to brain targeting drug delivery in recent years. One of these research areas was intranasal drug delivery.

Much interest has been given to the exploitation of the nasal route for the delivery of drugs to the brain via the olfactory mucosa since the olfactory nerve cells are in direct contact with both the environment and the CNS. Recently, several examples of nasal drug delivery to the brain have been reported. Small molecular, drugs such as cocaine and benzoylecgonine (Chow et al., 1999, 2001), local anesthetics (Chou and Donovan, 1998), dihydroergotamine (Wang et al., 1998) and dopamine (Dahlin et al., 2001) have been shown to reach brain via the olfactory pathway in animals. Illum (2000) has reviewed the transport of drugs from the nasal cavity to CNS. Recently, several nasal formulations such as ergotamine (Novartis), sumatriptan (GlaxoSmithKline) and zolmitriptan (AstraZeneca) have been marketed to treat migraine.

* Corresponding author. Tel.: +86-21-5423-7381; fax: +86-21-5423-7381.
E-mail address: xgjiang@shmu.edu.cn (X. Jiang).

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Especially, Thorne and Frey (2001) and Frey (2002) reviewed the latest progress of delivering neurotrophic therapeutic agents into CNS via nasal route. We could expect the appearance of nasally administered neurotrophic factors to treat CNS diseases in future.

Methotrexate (MTX), a folic acid antagonist, is used extensively against a variety of systemic malignant disorders. However, intravenous (i.v.) injection of MTX has not proven to be equally effective against malignancies involving CNS. This is presumably due to the poor penetration of intact MTX across the BBB (Rall, 1965). For clinical use, the drug is usually injected into the lumbar subarachnoid space either alone or with other anti-tumor drugs. By intrathecal administration, a therapeutic level of MTX in CSF could be quickly attained and be maintained for a relatively long time. On the other side, MTX intrathecal injection sometimes causes series of adverse effects such as progressive paraplegia, Balint's syndrome, anemia, long-term cerebral metabolite changes, etc.

Since MTX i.v. administration cannot treat CNS malignancy, such as acute CNS leukemia, and intrathecal injection requires specific lumbar puncture and could result in many side effects; neither of these routes is optimal for clinical use. In contrast, nasal administration can provide more convenient and non-invasive patient self-administration. If MTX nasal administration can attain therapeutic levels in CSF, it is possible to become a novel means for certain brain cancers' prophylaxis and treatment.

The aim of this paper was to investigate the concentration profiles of MTX in blood and CSF after intranasal (i.n.) and i.v. administration in rats. Consequently, we could find out whether there exists a direct nose–brain pathway into the CSF following i.n. administration. Also we find the degree of drug targeting to CSF attained following MTX intranasal delivery.

2. Materials and methods

2.1. Drugs and chemicals

MTX raw material was obtained from Zhejiang Wanma Pharmaceutical Co. Ltd., China. Methotrexate sodium was obtained from Shanghai Hualian Pharmaceutical Co. Ltd., China. Urethane (Shanghai Chemical Reagents Corp., China) was used as anesthetics for animal experiments. Millipore ultrapure deionized water (Millipore Simplicity^{TM}, USA) was used for preparation of the solutions and buffer of HPLC mobile phase. HPLC grade methanol was purchased from Shanghai Chemical Reagents Research Institute, China. All other chemicals were of commercially analytical grade.

2.2. Animal preparation

The animal experiment was carried out in compliance with the protocol of Animal Use and Care by Medical Center of Fudan University. Male Sprague–Dawley rats (270–330 g) were anesthetized with an intraperitoneal dose of 25% (w/v) urethane (1.5 g/kg) and kept under anesthesia throughout the whole experiment. An incision was made along the neck, the trachea was severed and the upper part was tied off with a suture, the lower part was cannulated with a PE tubing to aid air breathing. The anesthetized animals were placed on a warming pad to maintain normal body temperature.

To insure against drainage of nasally dosed solution, the nasal cavity was isolated from the respiratory and gastrointestinal tracts by tying off the upper part of esophagus, rather than sealing the nasopalatine opening using certain adhesive (Hussain et al., 1990). As Illum (2000) illustrated, in the usually used nasal cavity isolation techniques, the trauma due to invasive surgery and the rapid sealing effect of cyanoacrylate glue inevitably influence the characteristics of the drug transport process. In our experiments, nasal cavity was isolated by tying off the upper part of esophagus. Thus, there are fewer traumas in order that the drug transport can took place under more physiological condition.

The anesthetized animal was then moved to a stereotaxic apparatus (ST-7-8111, NARISHIGE, Japan). The ear bars of the apparatus were placed into the rats' ears and the chin was tilted forward toward the chest. The cisterna magna was exposed by making a blunt dissection through the allanto-occipital muscle. The rat's head was then moved to a horizontal position. The lateral ventricle was located according to KK atlas. From the following stereotaxic coordinates: posterior 0.9 mm and lateral 1.5 mm from the bregma, a hole was drilled through the skull, a short piece of

head-polished needle (0.3 mm diameter) attached to a segment of PE-20 tubing was inserted to a depth of 3.5 mm to locate the lateral ventricle. A syringe pump (DHL-A, Shanghai Huxi Analytical Apparatus, Shanghai, China) was attached to the end of the PE-20 tubing. It is used to infuse artificial CSF (NaCl, 127 mM; KCl, 2.5 mM; $MgCl_2$, 0.9 mM; Na_2HPO_4, 1.2 mM; $CaCl_2$, 1.3 mM; $NaHCO_3$, 21 mM; glucose, 3.4 mM, pH 7.3) into the lateral ventricle. A 25 gauge needle was placed into the cisterna magna to collect CSF samples. The volume of CSF withdrawn with each sample was replaced by an infusion of same volume of artificial CSF into the lateral ventricle (Chou and Donovan, 1997). The infusion rate of artificial CSF was 10 μl/min during the first one and a half hours and 5 μl/min during the remaining time (stop infusing, once the infused amount of artificial CSF reaches 60 μl during each sampling interval). The stopping time intervals allow the balance of MTX between artificial CSF and brain. In this way, CSF samples could be obtained continuously throughout the entire experiment.

2.3. Experiments

2.3.1. Nasal absorption studies

MTX nasal spray (12.5 mg/ml) was prepared by the following procedures: MTX raw drug was dissolved by adding Na_2CO_3 aided by ultrasonic dispersion, phosphate buffer was added to maintain the proper pH, reductant Na_2SO_3 and antiseptic benzyl alcohol were added. A dose (3.2 mg/kg) was administered to five rats into nostrils by carefully inserting a length of PE-10 tubing attached to a micropipette.

After tying off the esophagus, MTX was administered at a dose of 3.2 mg/kg to each rat over a 30-s period. A volume (128 μl/kg) of drug solution was placed into each nostril by carefully inserting a length of PE-10 tubing attached to a micropipette.

Prior to and at certain time points (5, 10, 15, 30, 45, 60, 90, 120, 150, 180, and 240 min) after MTX intranasal administration, from the caudal vein 200 μl of blood samples were collected into heparinized polyethylene conical tubes, and centrifuged 10 min at 8000 rpm for 80 μl plasma at 15, 30, 45, 60, 75, 90, 120, 150, 180, and 240 min after MTX nasal administration, CSF samples of 60 μl were collected from the cisterna magna without heparinization. Both plasma and CSF samples were stored at −20 °C.

2.3.2. Intravenous administration

MTX drug solutions (1.0 mg/ml) were prepared in saline for i.v. injection, an equivalent dose (3.2 mg/kg) to i.n. route were administered via the femoral vein. The plasma and CSF samples were collected at the same time points as the i.n. route.

2.3.3. Analytical procedures

All MTX concentrations in the plasma and CSF were measured by HPLC. CSF and plasma samples were thawed prior to HPLC analysis. CSF samples were analyzed just after centrifuging at 10,000 rpm for 5 min without further treatment. Plasma samples were treated to precipitate the serum proteins as the following procedures: a volume of 80 μl plasma was placed into a 0.6 ml polyethylene conical centrifuge tube, 80 μl 10% perchloric acid (v/v) were added into the tube, the mixture was vortex-mixed violently for 2 min and then centrifuged at 10,000 rpm for 10 min. The 50 μl supernatant was injected directly into a C_{18} column (Diamonsil, 20 cm × 4.6 mm, 5 μm, Dikma, USA). The mobile phase was composed of a mixture of 50 mM ammonium acetate buffer (pH 6.0) and methanol (77:23, v/v) with a flow rate of 1.0 ml/min. The ultraviolet absorbance of the effluent was monitored at a wavelength of 313 nm. The chromatographic system consisted of a LC-10AT VP solvent delivery system, and SPD-10A UV spectrophotometric detector (Shimadzu, Kyoto, Japan), and HS 2000G chromatographic integrator (HS Empire, Hangzhou, China).

The HPLC method used above was rapid and sensitive for measuring MTX in rat plasma and CSF. The limit of quantitation for MTX was found to be 2.5 ng, which corresponds to a concentration of 50 ng/ml for plasma and 25 ng/ml in CSF with an injection volume of 50 μl.

2.3.4. Data analysis

Results obtained from the HPLC analyses were plotted as drug concentration versus time curves in CSF or plasma. The AUC values for each curve were calculated from the time zero to the last data point using the stand trapezoidal method without extrapolation to infinity.

The bioavailability was calculated by dividing the mean plasma AUC after nasal dosing by the mean value after i.v. administration. The apparent CSF

availability was defined as the ratio of $AUC_{CSF,i.n.}/AUC_{CSF,i.v.}$. According to Hunt et al. (1986), the degree of MTX targeting to CSF after intranasal administration can be evaluated by the drug targeting index (DTI), which can be described as the ratio of the value of AUC_{CSF}/AUC_{plasma} following intranasal administration to that following intravenous injection. The higher the DTI is, the further degree of MTX targeting to CSF can be expected after intranasal administration.

$$DTI = \frac{(AUC_{CSF}/AUC_{plasma})_{i.n.}}{(AUC_{CSF}/AUC_{plasma})_{i.v.}}$$

Statistical differences between i.n. and i.v. administration were concluded using the unpaired Student's *t*-test and a value of $P < 0.05$ was considered statistically significant. Results are presented as mean values ± S.D.

3. Results

MTX concentration profiles in plasma and CSF following i.v. or i.n. administration are shown in Figs. 1 and 2. It was found that the MTX levels in CSF of nasal route were significantly higher than those obtained after i.v. injection despite the much lower MTX concentrations in plasma of nasal route than those of i.v. injection. From the time of 30–180 min, very significant differences of concentrations in CSF were observed between i.n. and i.v. routes according to the Student's *t*-test ($P < 0.01$).

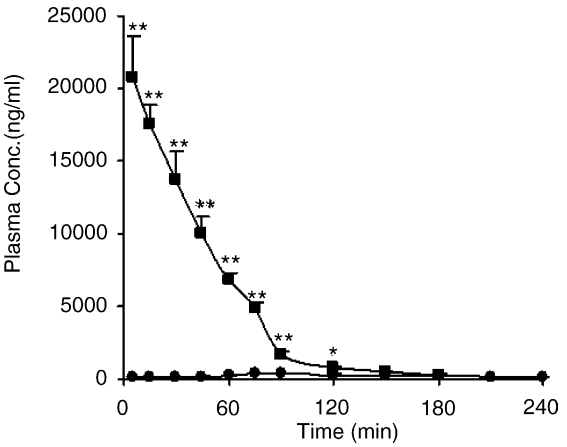

Fig. 1. MTX concentrations in plasma following intravenous (■) and intranasal (●) administration (dose: 3.2 mg/kg). Data represent the mean ± S.D. ($n = 5$). Any significant difference at each time point between two routes was calculated according to the Student's *t*-test: (⋆): $P < 0.05$; (⋆⋆): $P < 0.01$.

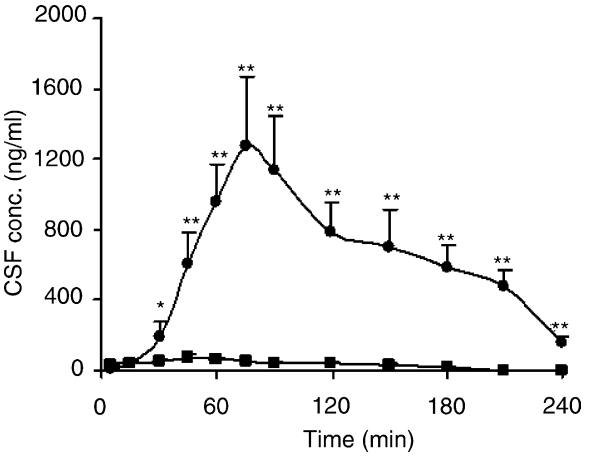

Fig. 2. MTX concentrations in CSF following intravenous (■) and intranasal (●) administration (dose: 3.2 mg/kg). Data represent the mean ± S.D. ($n = 5$). Any significant difference at each time point between two routes was calculated according to the Student's *t*-test: (⋆): $P < 0.05$; (⋆⋆): $P < 0.01$.

At 5 min following i.v. injection, MTX attained a high concentration of 20,715 ± 2938 ng/ml, and then followed by an exponential decline depending on the time. Comparatively, nasally applied MTX displayed a slow and poor absorption across the nasal mucosa into the systemic circulation, 90 min after nasal administration, MTX plasma concentration reached a peak value of 345 ± 58 ng/ml, far lower than the maximal plasma concentration of i.v. injected MTX, from the period of 5–120 min, MTX in plasma after i.n. administration were all significantly lower than those after i.v. injection (very significant, $P < 0.01$).

However, the MTX concentrations in CSF of two routes were just the contrary. Following i.n. administration, the MTX levels in CSF were significantly higher than those obtained after i.v. injection. From the time periods of 30–240 min, significant differences of concentrations in CSF were observed between i.n. and i.v. routes ($P < 0.01$). At 75 min after nasal dosing, MTX concentration in CSF reached a peak value of 1278 ± 393 ng/ml, and then followed by a prolonged duration of MTX in CSF, which had exceeded the MTX therapeutic level in CSF. A MTX

Table 1
AUC values of MTX following intranasal and intravenous administration

Route	AUC (ng min/ml)		Ratio of AUC_{CSF}/AUC_{plasma}
	Plasma (mean ± S.D.)	CSF (mean ± S.D.)	
Intranasal	49227 ± 13293	104844 ± 35718	2.13
Intravenous	779835 ± 73571	7620 ± 1062	0.098
Ratio of $AUC_{i.n.}/AUC_{i.v.}$	6.3%[a]	13.76%[b]	DTI[c] = 21.7

[a] Bioavailability.
[b] Apparent CSF availability.
[c] Drug targeting index.

level in CSF of 1 μM has been suggested as an effective level against meningeal leukemia and carcinomatosis (Shapiro et al., 1975). In contrast, MTX concentrations in CSF after i.v. injection never exceeded 100 ng/ml, the peak value in CSF was only 79 ± 12 ng/ml, quantitatively explained the poor CNS therapeutic effect of MTX injection.

The AUC of drug concentration curves in plasma and CSF were calculated, the results were shown in Table 1. The bioavailability of MTX obtained following nasal administration was only 6.3%; the apparent CSF availability of nasally applied MTX was 13.8 (Table 1). As for the DTI, the value achieved was 21.7, far greater than 1. We can observe a measurable degree of MTX targeting to CSF following intranasal administration.

These findings suggested that the existence of an alternative transport pathway to the CSF other than the penetration across the BBB from the systemic circulation. MTX nasally administered has a characteristic of brain targeting; it may be helpful for both increasing the CSF therapeutic levels and reducing the systemic side effects.

4. Discussion

Methotrexate is an antimetabolite used in the treatment of certain neoplastic diseases, severe psoriasis, and adult rheumatoid arthritis. The widely used MTX, has good therapeutic effects against various kinds of tumors, such as acute leukemia, chest cancer, when applied systemically; if administered intrathecally or by intraventricular injection, it can also treat some CNS neoplasm.

The delivery of therapeutic molecules into the CNS has proven to be a major obstacle in treating brain disorders. The BBB can be a major impediment to the treatment of diseases of the CNS, as many drugs are unable to reach there at therapeutic concentrations.

The nose–brain pathway, as a conduit for transport of agents into the CNS, is an area of ongoing research. Many substances, including viruses, metals, dyes, small molecular drugs, proteins and hormones, have been reported to gain direct access to the CSF and/or brain following nasal administration (Mathison et al., 1998).

Some lipophilic drugs, such as progesterone (Kumar et al., 1982), which can easily go across the BBB didn't show any expected increased uptake in CSF after nasal administration. The maximum CFS levels obtained following i.v. injection, i.v. infusion, and nasal spray of the same dose (10 μg) were 0.23, 0.66, and 0.53 nM, respectively. Increased drug concentration in CSF was not observed. Hussain explained the rapid and full absorption across nasal mucosa into systemic circulation made any transport via the olfactory pathway into CNS insignificant (Hussain et al., 1990).

On the other hand, the direct pathways for the transfer of low BBB permeability drugs from the nasal cavity via the olfactory mucosa into the CNS can be supported. Sakane et al. (1991a) reported that cephalexin was preferentially to enter CSF after nasal administration as compared to i.v. and intraduodenal administration in rats. The levels in CSF were 166-fold higher 15 min after nasal administration than those of the other two routes. This is also the case with MTX. In our study, the AUC_{CSF} ratio of i.n. dosing was more than 13 times as high as i.v. injection. Illum illustrated that the direct pathway from nose–brain

may only be significant for compounds that are poorly absorbed from the nasal cavity to the systemic circulation or have low BBB transport properties (Illum, 2000). Here we can conclude that, for the poor BBB penetration drugs, it is promising to obtain high CSF drug concentrations through nasal drug delivery.

Sakane et al. (1991b) demonstrated that the transport of sulfonamides, relatively hydrophilic compounds, from the nasal cavity into the CSF was dependent on their lipophilicity; the extent of their absorption into the CSF could be predicted by the pH-partition theory (Sakane et al., 1994). MTX, $\log P = -1.85$, a relatively hydrophilic drug, obtained unexpected high $AUC_{CSF,i.n.}/AUC_{CSF,i.v.}$ ratio not corresponding his low octanol/H_2O distribution coefficient after nasal administration. It showed that the pH-partition theory might only work in predicting for relatively lipophilic drugs, similar results can be seen by the studies of cephalexin (Sakane et al., 1991a) and procaine (Chou and Donovan, 1998), both hydrophilic drugs.

Another interesting thing to be mentioned was that MTX showed a slow absorption into the systemic circulation and the CSF following i.n. administration, the t_{max} values in plasma and CSF of were 90 and 75 min, respectively, not as fast as cocaine (Chow et al., 1999) and dopamine (Dahlin et al., 2001). This may be owing to its limited penetration across the BBB and poor absorption into the systemic circulation via the nasal mucosa, MTX tended to accumulated in the olfactory mucosa and slowly transported into the CSF. Due to its hydrophilicity, we presumed MTX was reluctant to be absorbed by olfactory mucosa so as to be transported into CSF slowly. But to know how on earth MTX entered the CSF and other part of CNS, a more detailed research about the spatial distribution of MTX following nasal administration is required.

In some researches such as cephalexin, zidovudine (Seki et al., 1994), the CSF or brain drug concentrations usually were determined at one or two time points following drug administration. The existence of direct nose–brain pathway was judged by the CSF and plasma drug concentrations at that time. As Chow mentioned, the evidences seemed insufficient, it was not according to the comparison between peak concentrations of two administration routes. The method of CSF sampling accounted for this limitation. In those studies, CSF samples were collected by cisternal puncture at single time, to examine the complete drug disposition in CSF, one time point may require five rats and what's more, the differences between individual rats could not be eliminated. By using the artificial CSF perfusion technique in our experiment, we could collect the CSF sample continuously and by using the same rat, the full drug concentration profile in CSF and in plasma could be obtained. So in this research, a more complete pharmacokinetic examination of drug levels in plasma and CSF in the rat has been carried out to verify the existence of a direct nose–brain pathway of nasally applied MTX.

The supine or prone position of rat in nasal absorption experiments has a large influence on the drug absorption across the nasal mucosa and consequent distribution in blood, CSF. Generally, the supine position can assure fully contact with a larger area of nasal mucosa and have a better absorption into systemic circulation than the prone position. In our experiments, due to the limitation of stereotaxic apparatus, all rats took the prone position in both intravenous and intranasal administration. Although nasally applied MTX may obtain lower bioavailability, we can still make judgment according to their AUC_{CSF}/AUC_{plasma}, from Table 1; we can see the AUC_{CSF}/AUC_{plasma} ration of nasal route was 2.13, far greater than the ratio of 0.098 after intravenous administration. The targeting therapeutic regions of nasally applied MTX could be CSF and surface brain tissues. The acute CNS leukemia and meningeal tumor may be suitable models to testify the efficacy of MTX nasally administered. Shingaki et al. (1999) evaluated the in vivo therapeutic potency of nasally administered MTX by using 9L glioma cell-bearing rats. Compared to the control non-treated group and intraperitoneal group, the tumor weight of the MTX nasally treated group was significantly reduced. The pharmacodynamic evidence also supported the existence of direct transport from the nasal cavity to CSF.

Rats were the most-frequently used animal model in the nasal route for brain targeting researches, the anatomical differences between rats and human being should be considered. It's true that the olfactory and respiratory epithelia of the rat are interspersed throughout the entire nasal mucosa, while in human the olfactory epithelium is present only at the roof of the nasal cavity. It seems disadvantageous for the drug transport into CNS via the olfactory pathway when

applied in human, but there are also some advantageous aspects, the turnover of the CSF in human is slow (four to five times per day), which can lead to higher drug concentrations in CSF, moreover, the CSF volume and turnover may decrease with age, a higher CSF drug concentration could be more easily achieved in elderly patients. From the perspective of drug formulations, using viscous solutions or gels for nasal dosing may succeed in prolong the retention time of drug over the olfactory areas; also we can use some devices (e.g. through the soft tube inserted into the human nasal cavity to the olfactory areas) to apply the drug to the olfactory areas.

5. Conclusion

The MTX concentrations in CSF achieved after i.n. administration were significantly higher than those after i.v. administration ($P < 0.01$). The ratio of the AUC_{CSF} value between i.n. route and i.v. injection was 13.76, and the DTI value was 21.7. These results suggested that the antineoplastic MTX can be directly transported from the nasal cavity into the CSF in rats. So nasally administered MTX is promising to become an effective non-invasive route for certain brain cancers' prophylaxis and treatment, although it has not been clinically confirmed.

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