FULL PAPER *Pathology*

Effect of methotrexate exposure at late gestation on development of telencephalon in rat fetal brain

Ayano HIRAKO[1)], Satoshi FURUKAWA[2)], Takashi TAKEUCHI[1)] and Akihiko SUGIYAMA[1)]*

[1)]*Courses of Veterinary Laboratory Medicine, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Minami 4–101 Koyama-cho, Tottori, Tottori 680–8553, Japan*
[2)]*Toxicology and Environmental Science Department, Biological Research Laboratories, Nissan Chemical Industries, Ltd., 1470 Shiraoka, Shiraoka-shi, Saitama 349–0294, Japan*

(Received 30 June 2015/Accepted 2 September 2015/Published online in J-STAGE 13 September 2015)

ABSTRACT. Pregnant rats were treated with 30 mg/kg of methotrexate (MTX) on gestation day (GD) 16, and fetal brains were examined time-dependently. On GD 20, the appearance of the telencephalon in the MTX group was different from that in the control group, and the major axis of the telencephalon of the MTX group was shortened, compared to that of the control group. In the sagittal section of the telencephalon in the MTX group on GD 20, histopathological findings of deformation and narrowing of the cerebral ventricle, the disturbance of the arrangement of the marginal cell layer of subventricular zone (SVZ) and thickening of telencephalic wall, cortical plate and ventricular zone (VZ)/SVZ were possibly attributable to neuronal migration disorders by MTX. Through all the experimental period, few pyknotic cells or TUNEL-positive cells were observed in the VZ/SVZ of the telencephalic wall and striatum in the control group. On the other hand, in the VZ/SVZ of the telencephalic wall and striatum in the MTX group, pyknotic cells or TUNEL-positive cells were observed on GD 17, and they increased significantly on GD18 and then decreased to the control levels from GD 19 onward. The phospho-Histone H3-positive rate decreased remarkedly in the VZ/SVZ of the telencephalic wall and striatum of the MTX group on GDs 17 and 18, compared to the control group, but they recovered on and after GD 19. These results suggested that there was a high possibility that development of the telencephalon in this period required strong folic acid.

KEY WORDS: apoptosis, brain, fetus, methotrexate, neuronal migration disorder

doi: 10.1292/jvms.15-0389; *J. Vet. Med. Sci.* 78(2): 213–220, 2016

Folate is a water-soluble B-complex vitamin which functions as a coenzyme in single-carbon transfers in the metabolism of nucleic acid and amino acids [11, 47]. Folate plays an important part in the brain development in the prenatal periods [11, 40]. Folate deficiency during pregnancy is one of several well-established factors that can increase the risk of neural-tube defects, especially spina bifida and anencephaly [11, 40]. Folic acid deficiency during late gestation decreased the number of progenitor cells undergoing cell proliferation in the ventricular zones (VZ) of the septum, striatum (caudate putamen) and neocortex and increased apoptosis in the septum of fetal mouse brain [6]. Prenatal folate deficiency manifested later with increased anxiety 9–12 weeks after birth in mice [12]. As stated above, there have been several reports demonstrating the relation between folate deficiency in the prenatal period and abnormal development of brain. However, studies of the consequences of inadequate prenatal folate status on brain development of fetuses are scarce; detailed histopathological findings of fetal brains of folate-deficient animals have not been reported. Folate deficiency occurs in various pathophysiological conditions including impairment in intestinal absorption and renal tubular reabsorption induced by alcohol consumption, and intestinal diseases, such as celiac disease and renal malfunctioning [53]. Folate deficiency is also induced by chronic use of anticonvulsants, such as phenobarbital, promidone, carbamazepine and valproate phenytoin [53].

A folate antagonist, methotrexate (MTX), prevents *de novo* pyrimidine and purine synthesis, required for DNA and RNA synthesis, and consequently inhibits cell proliferation [54]. MTX is known to induce apoptosis, and the mechanism of MTX-induced apoptosis is considered to be associated with the up-regulation of p53 and p21 proteins [24, 46], repression of the induction of c-Jun N-terminal kinase (JNK) activity [46], expression of the CD95 receptor/ligand system [36] and reactive oxygen species [20, 46]. MTX has been used in the treatment of malignant tumor, autoimmune and inflammatory diseases, and gestational trophoblast disease [8, 9, 17]. In human, MTX induced brain malformation, such as alobar/semilobar holoprosencephaly [5, 43], cerebellar hypoplasia [43], agenesis of the corpus callosum [43] and microcephaly [2, 16].

In the normal rat brain development, neural progenitor cells proliferate in the VZ and subventricular zone (SVZ), after which they differentiate into neurons [38]. Postmitotic neurons migrate out of the VZ/SVZ and accumulate to form the cortical plate, subplate and marginal zone [38]. The cortical plate neurons are generated in an inside-out gradient and produce a laminar structure [38]. On gestation day (GD) 16 in

*CORRESPONDENCE TO: SUGIYAMA, A., Courses of Veterinary Laboratory Medicine, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Minami 4–101 Koyama-cho, Tottori, Tottori 680–8553, Japan. e-mail: sugiyama@muses.tottori-u.ac.jp

rats, neural progenitor cells proliferate in VZ/SVZ and pass through the intermediate zone, move to the side of meninges and formed the subplate and cortical plate [27, 37, 44]. MTX exposure on GD 13 in rats induced apoptosis and inhibited cell proliferation of neuroepithelial cells in the VZ of telencephalon [49]. However, the detailed histopathological changes of the telencephalon following MTX administration to dams on GD16 have not been previously reported. Therefore, in the present study, we examined histopathologically the time-dependent changes of fetal brain following MTX treatments on GD 16, to clarify effect of MTX on the telencephalon development including neuronal migration and to determine the significance of folate on the telencephalon development in rat fetus. The significance of the present study is to elucidate the unsolved mechanism in the pathogenesis of abnormal brain development induced by MTX or folate-deficiency, by clarifying the effect of MTX exposure on GD 16, the period during which proliferation and migration of neural progenitor cells occur on brain development.

MATERIALS AND METHODS

Pregnant rats were treated with 30 mg/kg of MTX on GD16, and fetal brains were examined histopathologically on GDs 17, 18, 19 and 20.

Animals: All experiments were performed using female Wistar Imamichi rats, 9–10 weeks of age, 305.7 ± 4.1 g (mean ± SE) in weight, and obtained from the Institute of Animal Reproduction (Kasumigaura, Japan). The animals were reared in a room with the temperature controlled at 22 ± 2°C, humidity at 50 ± 5%, with ventilation 11 times per hour, lighting at 12:12-hr light/dark cycle (light cycle, 7:00–19:00) and given standard chow (CE-2; CLEA Japan, Inc., Tokyo, Japan). The present experiments were performed following the provisions approved by the Animal Research Committee of Tottori University.

Experimental design: A total of 24 animals were divided into two groups as follows: (1) saline-treated control rats (n=12) and (2) MTX-treated rats (n=12). MTX (Pfizer Japan Inc., Tokyo, Japan) was dissolved in saline. Day 0 of gestation (GD 0) was designated as the day when the presence of a vaginal plug was identified. The rats received intraperitoneal injections (i.p.) with MTX (30 mg/kg body weight) or saline (the control) on GD 16. The specific timing of MTX administration was selected, because GD 16 is the period when neuronal progenitor cells proliferated in VZ/SVZ and then move from VZ/SVZ to the side of meninges to form the subplate and cortical plate [37, 44]. In our preliminary study, although MTX 5 and 15 mg/kg treatment on GD 16 induced few histopathological changes in the VZ/SVZ of the telencephalic wall and striatum in fetal brain, MTX 30 mg/kg treatment on GD 16 induced pyknotic changes in the same regions of rat fetal brain.

Fetus samples were collected after euthanasia by overdose administration of pentobarbiturate (100 mg/kg, i.p.) on GDs 17, 18, 19 and 20. They were then removed from the uterus and weighed.

Histopathological examination: For histopathological examination, all fetuses were fixed in 10% neutral buffered formalin and then embedded in paraffin. The fetal brain tissues were sectioned, stained with hematoxylin and eosin and examined with light microscopy.

TUNEL method: DNA-fragmented neuronal cells in telencephalon and striatum were detected by terminal deoxynucleotidyl-transferase (TdT)-mediated deoxyuridine triphosphate-digoxigenin (dUTP) nick-end labeling (TUNEL), which was performed using an *in situ* apoptosis detection kit (Trevigen, Inc., Gaithersburg, MD, U.S.A.). The number of TUNEL-positive cells was obtained from over 500 neuronal cells in the VZ/SVZ of the telencephalic wall and striatum for each fetus by light microscopy, and the TUNEL-index was calculated as the percentage of TUNEL-positive cells out of the total number of neuronal cells counted.

Immunohistochemical examinations of rat fetuses: Immunohistochemical stain was performed by a labeled-polymer method using Histofine Simple Stain MAX-PO (R) (Nichirei, Tokyo, Japan). To retrieve the antigen, tissue sections for the detection of phospho-histone H3 antigen were immersed in citrate buffer, pH 6.0 (Dako, Glostrup, Denmark) and microwaved for 15 min at 500 W (NE-EH22/ Panasonic Corp., Tokyo, Japan). Endogenous peroxidase activity was quenched by immersing the sections in 3% hydrogen peroxide in methanol for 15 min. The sections were incubated with the phospho-histone H3 rabbit monoclonal antibody (1:1,500 dilution; Abcam, Tokyo, Japan) for 30 min at room temperature and then treated with Histofine Simple Stain MAX-PO (R) for 30 min at room temperature. They were exposed to a 3,3′-diaminobenzidine solution containing hydrogen peroxide (Nichirei) to facilitate a peroxidase color reaction and then counterstained with Mayer's hematoxylin. The number of phospho-histone H3-positive cells was counted from over 500 neuroepithelial cells in the VZ/SVZ of the telencephalic wall and striatum for each fetus by light microscopy, and the phospho-histone H3-index was calculated as the percentage of phospho-histone H3-positive cells out of the total number of neuronal cells counted.

Statistical analysis: Means ± standard error (SE) of the individual litter value was calculated. Comparisons between the two groups were made by Student's t-test or Welch's t-test if data were normally distributed, and by Mann-Whitney U test if data were not normally distributed with statistical software ("Excel Toukei 2008", SSRI Co., Ltd., Tokyo, Japan). The data were analyzed with an F-test. When variances were homogenous, the Student's t-test was performed. Welch's t-test was employed when variances were not homogeneous (P<0.05). P<0.05 or P<0.01 was considered to be statistically significant.

RESULTS

MTX effects on rat fetuses: On GDs 17, 18 and 19, the dead fetus ratio was 0% both in the control group and the MTX group (Table 1). On GD 20, the total number of live fetuses in the MTX group decreased compared to the control group (Table 1). On GD 20, the number of living fetuses per litter showed a tendency to decrease, and the dead fetus

Table 1. Effect of MTX on rat fetuses

	Treatment	No. of dams	Total No. of live fetuses	Living fetuses per litter	Dead fetus ratio (%)
GD17	Control	3	43	14.3 ± 0.7	0
	MTX	3	46	15.3 ± 0.7	0
GD18	Control	3	45	15.0 ± 1.5	0
	MTX	3	47	15.7 ± 0.9	0
GD19	Control	3	48	16.0 ± 1.0	0
	MTX	3	42	14.0 ± 1.0	0
GD20	Control	3	43	14.3 ± 1.2	0
	MTX	3	27	9.0 ± 1.5	26.9 ± 15.4

Values are expressed as means ± SE.

ratio showed a tendency to increase, compared to the control group (Table 1).

Macroscopical and microscopical findings of rat fetal brain: On GDs 17, 18 and 19, there were no differences between the appearances of the telencephalon in the MTX group and those in the saline group. However, On GD 20, the appearance of the telencephalon in the MTX group was different from that in the control group (Figs. 1 and 2). While the telencephalic length showed a tendency to be greater than the telencephalic width in the control group on GD 20, the telencephalic width showed a tendency to be greater than the telencephalic length in the MTX group on GD 20 (Fig. 1). The longitudinal fissure of telencephalon in the MTX group on GD 20 showed a tendency to be shorter, compared to the control group on GD20 (Fig. 1). The telencephalic length of the MTX group (3.79 ± 0.07 mm) was significantly shortened compared to that of the control group (4.21 ± 0.07 mm)(Value is mean ± SE. Significantly different from the control at $P<0.05$, Student's *t*-test). The sagittal section of telencephalon in the MTX group on GD 20 showed the deformation and narrowing of the cerebral ventricle (Fig. 2), thickening of telencephalic wall, cortical plate, intermediate zone and VZ/SVZ (Fig. 3) and the disturbance of the arrangement of the marginal cell layer of SVZ (Fig. 4), compared to the control group.

On GD18, both the thickness and the ratio of the VZ/SVZ in the MTX group were significantly decreased compared to those in the control group, and the telencephalic wall showed a tendency to be thinner compared to that in the control group (Table 2). The telencephalic wall and cortical plate in the MTX group were significantly thicker than those in the control group on GDs 19 and 20, and the VZ/SVZ of the MTX group was thicker than that of control group on GD 20 (Table 2). The ratio of VZ/SVZ layer thickness to the thickness of all layers of the telencephalic wall was significantly higher than that of the control group (Table 2).

Throughout the experimental period, few pyknotic cells or TUNEL-positive cells were observed in the VZ/SVZ of the telencephalic wall and striatum in the control group (Figs. 5, 6 and 9). On the other hand, in the VZ/SVZ of telencephalic wall and striatum in the MTX group, there were many pyknotic cells and TUNEL-positive cells on GDs 17 and 18 (Figs. 5 and 6). On GDs 17 and 18, TUNEL indices in the VZ/SVZ in the telencephalic wall and striatum of the MTX group were significantly higher than those of the control group (Fig. 9). TUNEL indices in the telencephalic wall and striatum of the MTX group decreased to the control levels from GD 19 onward (Fig. 9).

Many mitotic and phospho-histone H3-positive neuronal cells were located along the ventricular layer of the telencephalic wall in the control group, while there were fewer phospho-histone H3-positive cells on GDs 17 and 18 in the same region of the MTX-treated group than those in the control group (Fig. 7). Many mitotic and phospho-histone H3-positive neuronal cells were scattered diffusely in the SVZ of the striatum of the control group, while there were fewer phospho-Histone H3-positive cells in the SVZ of the striatum of the MTX group, compared to those of the control group on GDs 17 and 18 (Fig. 8). Although phospho-histone H3 indices decreased in the VZ/SVZ of the telencephalic wall and striatum in the MTX group on GDs 17 and 18, compared to those in the control group, they recovered from GD 19 onward (Fig. 9).

DISCUSSION

The development of the mammalian cerebral cortex consists of three steps: (1) production of neuronal precursor cells, (2) migration to their laminar position and (3) differentiation and development of their morphological and functional properties [50]. Neuronal migration in the neocortex takes place for the greater part between the 8th and the 20th weeks of gestation in human [45] and between embryonic day 14 and postnatal day (PD) 5 in rats [39]. In the present study, MTX exposure on GD 16 induced a disturbance in the arrangement of the marginal cell layer of SVZ and the thickening of the telencephalic wall, cortical plate and VZ/SVZ in the fetal rat brain on GD 20. Previous studies revealed that cortical thickening or thickening of the cortical gray matter in cerebral cortex malformation was caused by neuronal migration disorders [19, 21, 22, 32]. In human brain malformation caused by neuronal migration disordars, blurred boundaries were observed between cortical gray and white matter [21]. It is possible that these histopathological findings were attributed to neuronal migration disorders induced by MTX. Neuronal migration disorders have been described in experimental animals exposed to neuroteratogens, such as methylnitrosourea [25], methylazoxymethanol-acetate (MAM-ac) [4], ethanol [35], cocaine [18, 29] and ionizing radiation [51].

Neuronal migration disorder is caused by (1) excessive neuron death and (2) reduction of radial fibers in the radial glias [4, 13, 14, 25]. In the present study, MTX induced severe neuron death in VZ/SVZ of the telencephalic wall. This histopathological finding is assumed to be strongly associated with the neuronal migration disorder observed in the present study. It is unclear whether or not MTX induced neuronal migration disorder through reduction of the radial glial fiber, in the present study. To our knowledge, there are no reports demonstrating the effect of MTX or folate deficiency on the radial glial fiber.

In the present study, apoptotic changes and cell proliferation inhibition in VZ/SVZ in the telencephalic wall were induced on GDs 17 and 18, resulting in thinning of

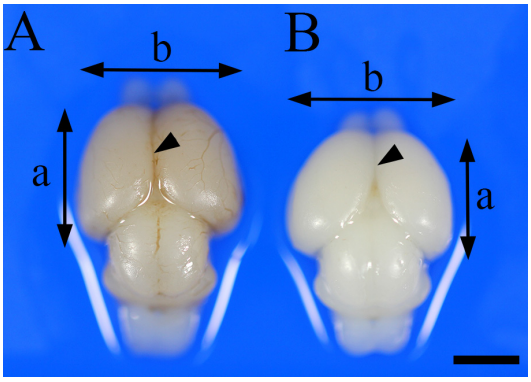

Fig. 1. Macroscopical findings of rat fetal brain in the control group (A) and the MTX-treated group (B) on GD 20. (a) Telencephalic length, (b) Telencephalic width. Arrowheads showed longitudinal fissure of telencephalon. Bar=3 mm.

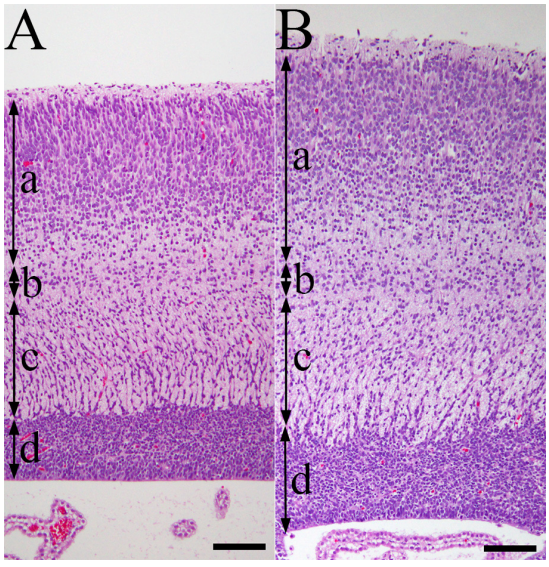

Fig. 3. Telencephalic wall in the control group (A) and the MTX-treated group (B) on GD 20. (a) Cortical plate, (b) Subplate, (c) Intermediate zone, (d) ventricular zone/subventricular zone. Bar=100 μm.

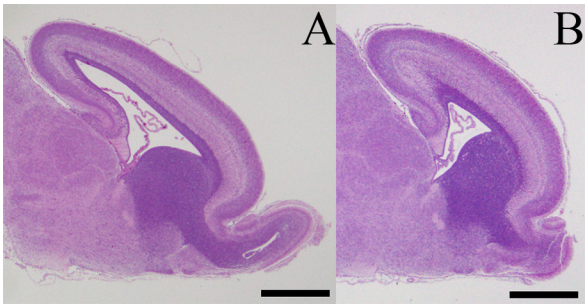

Fig. 2. Sagittal sections of telencephalon of rat fetal brain in the control group (A) and the MTX-treated group (B) on GD 20. Bar=1 mm.

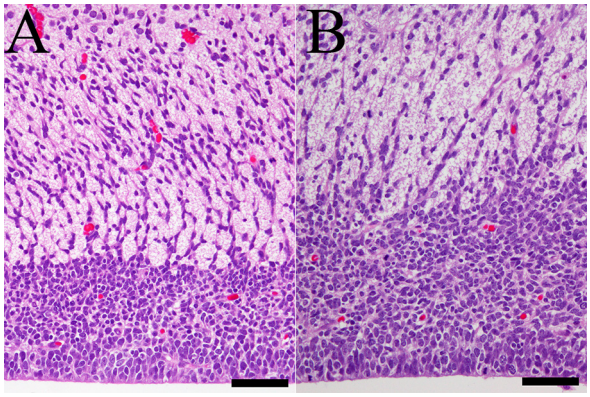

Fig. 4. Border region between subventricular zone and intermediate zone in cerebral cortex of the control group (A) and the MTX-treated group (B) on GD 20. Bar=50 μm.

VZ/SVZ in the telencephalic wall. However, the thickening of the telencephalic wall, cortical plate and VZ/SVZ in the telencephalic wall were observed on GD20. It was assumed that these histopathological changes participate largely in the recovery and increase of cell proliferation in VZ/SVZ of the telencephalic wall on GD 19, and they were the compensatory changes for preceding apoptosis and cell proliferation inhibition. These results are in agreement with the previous

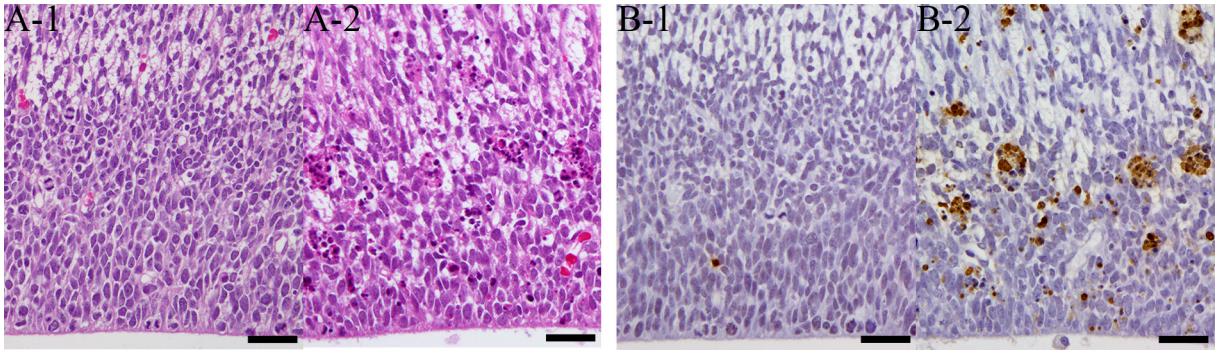

Fig. 5. Apoptotic changes in ventricular zone and subventricular zone in telencephalic wall of the control group (1) and the MTX-treated group (2) on GD 18. (A) HE stain, (B) TUNEL stain. Bar=30 μm.

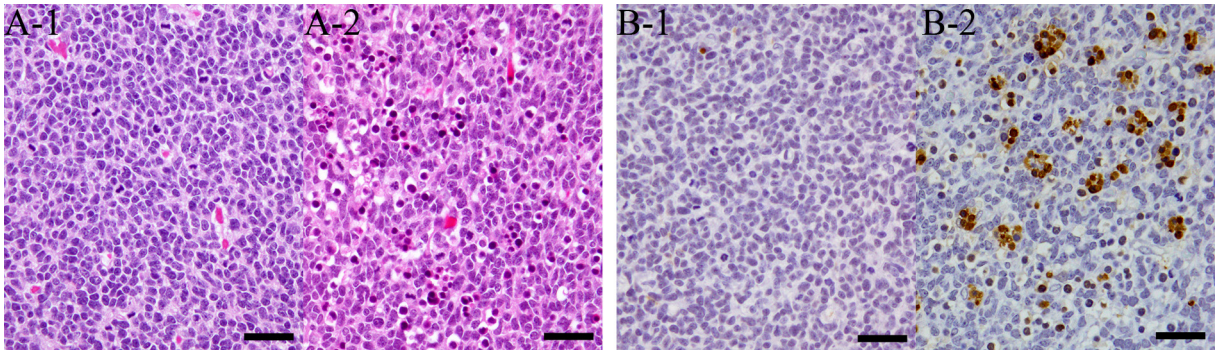

Fig. 6. Apoptotic changes in subventricular zone in striatum region of the control group (1) and the MTX-treated group (2) on GD 18. (A) HE stain, (B) TUNEL stain. Bar=30 μm.

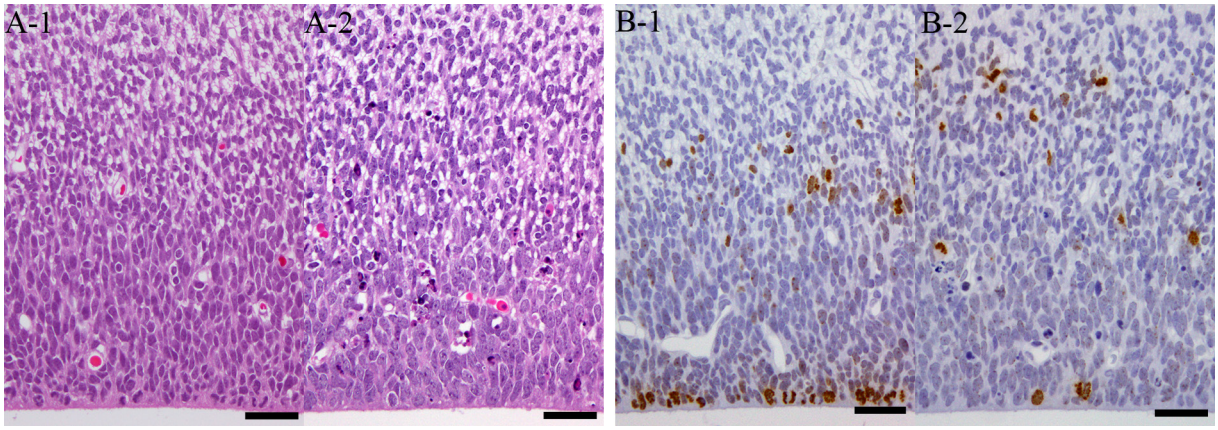

Fig. 7. Inhibition of cell proliferation in ventricular zone and subventricular zone in telencephalic wall of the control group (1) and the MTX-treated group (2) on GD 17. (A) HE stain, (B) Immunohistochemical stain for Phospho-Histone H3. Bar=30 μm.

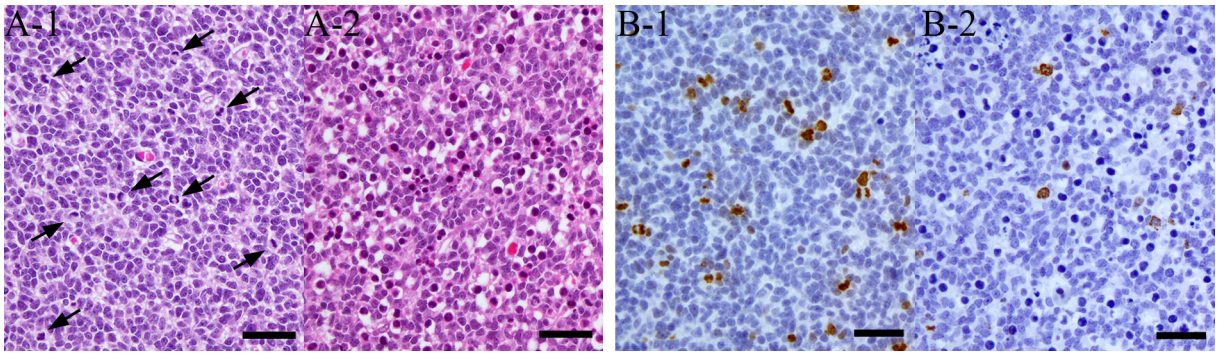

Fig. 8. Inhibition of cell proliferation in subventricular zone in striatum region of the control group (1) and the MTX-treated group (2) on GD 18. (A) HE stain, (B) Immunohistochemical stain for Phospho-Histone H3. Bar=30 μm. Arrows indicate mitotic figures.

studies [1, 48]. The MTX exposure on PD 6 in infant rats inhibited cell proliferation in the external granular layer of the cerebellum on PD 7, however, mitotic- and phospho-Histone H3-indices showed a tendency to increase compensatorily on PDs 8–10 [48]. Bálentová's study demonstrated that the prenatal exposure of the single whole-body dose of 3 Gy of gamma rays induced the increase in cell proliferation in the rostral migratory stream (RMS) in neonatal and young rats [1]. Bálentová *et al*. considered that the increase in cell proliferation in the RMS was related with radiation-induced genome instability as well as enhanced proliferating activity for compensation of the damaged cells loss [1]. Similarly to irradiation, MTX activates cell-cycle checkpoints and results in cell cycle arrest which allows DNA repair before cell proliferation or induces apoptosis [46]. In regulation of G1/S and G2/M transitions and preservation of genome

Table 2. Thickness of telencephalic wall, cortical plate and ventricular zone/ subventricular zone and ratio of each component of telencephalic wall

	Treatment	Telencephalic wall	Cortical plate	Subplate	Intermediate zone	Ventricular zone/ Subventricular zone
GD17	Control (μm)	523.66 ± 13.26	126.00 ± 2.69	19.07 ± 2.19	165.89 ± 8.36	182.90 ± 8.22
	(%)		(24.07 ± 0.20)	(3.63 ± 0.34)	(31.69 ± 1.47)	(34.94 ± 1.38)
	MTX (μm)	510.33 ± 3.33	121.62 ± 8.93	17.01 ± 1.20	172.93 ± 3.54	169.57 ± 4.27
	(%)		(23.82 ± 1.64)	(3.33 ± 0.22)	(33.88 ± 0.49)	(33.24 ± 0.99)
GD18	Control (μm)	641.87 ± 15.22	179.37 ± 14.62	25.37 ± 2.89	232.78 ± 10.39	181.44 ± 13.03
	(%)		(28.00 ± 2.28)	(3.96 ± 0.47)	(36.25 ± 1.16)	(28.23 ± 1.63)
	MTX (μm)	563.83 ± 30.20	173.98 ± 10.68	15.99 ± 2.19	25.13 ± 14.85	111.63 ± 5.13 [b]
	(%)		(30.85 ± 0.84)	(2.81 ± 0.26)	(41.70 ± 1.23 [a])	(19.87 ± 0.97 [a])
GD19	Control (μm)	698.78 ± 3.12	253.15 ± 2.12	24.05 ± 2.29	224.12 ± 6.00	168.96 ± 6.72
	(%)		(36.23 ± 0.39)	(3.44 ± 0.31)	(32.07 ± 0.72)	(24.18 ± 0.99)
	MTX (μm)	782.41 ± 10.95 [b]	292.21 ± 10.12 [a]	31.71 ± 6.63	257.85 ± 20.58	166.08 ± 10.39
	(%)		(37.39 ± 1.70)	(4.03 ± 0.78)	(32.90 ± 2.18)	(21.26 ± 1.57)
GD20	Control (μm)	743.09 ± 9.07	344.17 ± 4.10	25.52 ± 4.06	226.75 ± 15.85	117.33 ± 3.29
	(%)		(46.34 ± 1.07)	(3.45 ± 0.59)	(30.47 ± 1.79)	(15.78 ± 0.29)
	MTX (μm)	907.19 ± 19.46 [b]	397.86 ± 4.88 [b]	34.92 ± 1.56	258.51 ± 17.25	189.56 ± 17.40 [a]
	(%)		(43.87 ± 0.43)	(3.85 ± 0.16)	(28.54 ± 2.13)	(20.86 ± 1.65 [a])

a, b: Significantly different from control at $P<0.05$, $P<0.01$, respectively (Student's t-test).

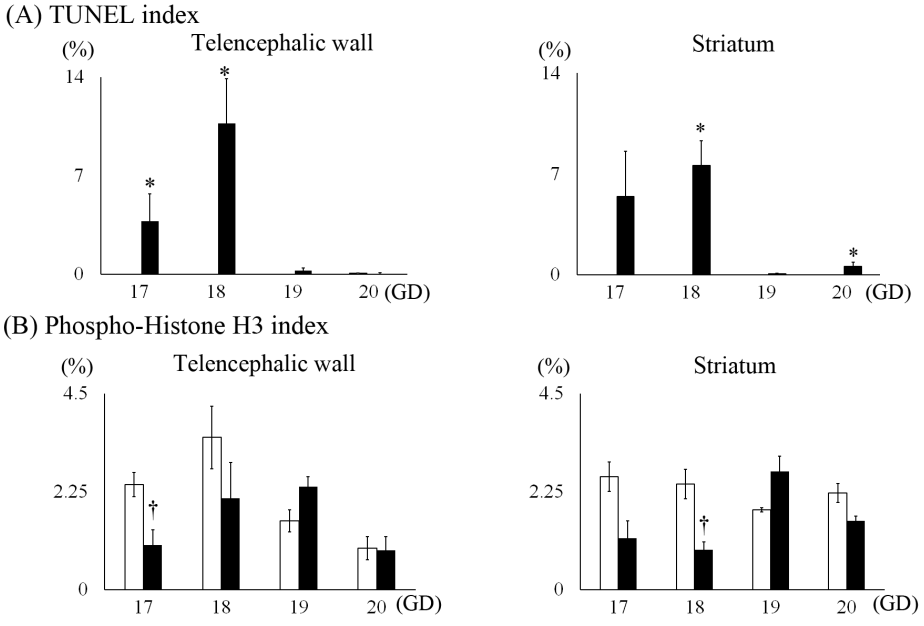

Fig. 9. Time course changes in the TUNEL indices (%) and the phospho-Histone H3 indices (%) in ventricular zone/subventricular zone of telencephalic wall and striatum region of rat fetal brain. Values are expressed as means ± SE. *: Significantly different from control at $P<0.05$ (Mann-Whitney's U-test). †: Significantly different from control at $P<0.05$ (Student's t-test).

integrity, the p53 protein plays an important role [26, 31]. Mutations of p53 gene, which are involved in the cell cycle and apoptosis control, mismatch repair of DNA and chromosome segregation control, can induce genome instability [26, 31]. To our knowledge, there has been no report demonstrating mutations of p53 gene induced by MTX. Hereafter, it is necessary to clarify the association between MTX treatment and p53 mutation.

In the present study, the deformation and narrowing of the cerebral ventricle were observed in the sagittal section of telencephalon in the MTX group on GD 20. Ventriculomegaly is well-known as cerebral ventricular malformation [33, 34, 52], and neuronal migration disorder is one of the causes of ventriculomegaly [15, 34]. Because the deformation and narrowing of the cerebral ventricle are categorized as cerebral ventricular malformation equivalent to ventriculomegaly,

neuronal migration disorder may also be the pathogenesis of the deformation and narrowing of the cerebral ventricle.

In the present study, severe apoptotic changes were induced by MTX in striatial VZ/SVZ. This region is also named ganglionic eminence, which produces GABAergic interneurons [10, 28]. GABAergic interneurons produced in ganglionic eminence migrate tangentially from ganglionic eminence to the telencephalic wall [10]. It was speculated that in the present study, severe apoptotic changes induced by MTX in striatial VZ/SVZ resulted in the decrease in GABAergic interneurons reaching the telencephalic wall, that may cause developmental retardation of telencephalic length.

Previous studies demonstrated that intraperitoneal injection of 3-nitropropionic acid, an irreversible inhibitor of succinate dehydrogenase of the mitochondrial complex II, induced apoptosis in the striatum of rodents [23, 41]. Treatment with 2.1% sevoflurane for six hr induced the up-regulation of activated caspase 3 and Bax expression in the striatum of seven-day-old rats [55]. Treatment with 0.75% isoflurane for four hr induced a significant increase in neuroapoptosis in the striatum of seven-day-old mice [3]. Folate-deficient diets from GD 11 to 17 decreased mitotic and phospho-histone H3-positive neural progenitor cells in the striatum of fetal mice on GD 17 [6, 7]. However, ours is the first report demonstrating the effect of folate metabolism antagonism or folate deficiency on the striatum development of fetal brain; no other reports show that. The striatum is composed of functional sub-units that are part of multiple cortico-striatal-thalamic circuits [30]. It has been recognized that the striatum contributes to the phenomena as follows: (1) learning of associations between stimuli, actions and rewards; (2) selection between competing response alternatives; (3) motivational modulation of motor behavior; and (4) declarative memory retrieval [30, 42]. In the present study, MTX exposure on GD 16 induced severe apoptotic changes in the SVZ of striatum of rat fetal brain. When the fetuses exposed to MTX on GD 16 are born and grow up, the above-mentioned functional disorders of the striatum may occur.

In conclusion, MTX administration of 30 mg/kg on GD 16 induced severe apoptotic changes and inhibited markedly cell proliferation in the VZ/SVZ of the telencephalic wall and the striatum. In the MTX-treated group, the disturbance of the arrangement of the marginal cell layer of SVZ and thickening of telencephalic wall, cortical plate and VZ/SVZ occurred, and it was considered that they were attributed to neuronal migration disorders induced by MTX. These results suggested that there was a high possibility that suitable amount of folic acid was required for telencephalon development in late pregnancy. This is, to our knowledge, the first research report clarifying the significance of the folic acid on telencephalon development in late pregnancy of rats. It now remains to verify whether or not folate supplementation reduces the MTX effect on telencephalon development.

REFERENCES

1. Bálentová, S., Raceková, E. and Misúrová, E. 2007. Effect of paternal exposure to gamma rays on juvenile rat forebrain. *Neurotoxicol. Teratol.* **29**: 521–526. [Medline] [CrossRef]
2. Bawle, E. V., Conard, J. V. and Weiss, L. 1998. Adult and two children with fetal methotrexate syndrome. *Teratology* **57**: 51–55. [Medline] [CrossRef]
3. Cattano, D., Williamson, P., Fukui, K., Avidan, M., Evers, A. S., Olney, J. W. and Young, C. 2008. Potential of xenon to induce or to protect against neuroapoptosis in the developing mouse brain. *Can. J. Anaesth.* **55**: 429–436. [Medline] [CrossRef]
4. Ciaroni, S., Cecchini, T., Gazzanelli, G. and Del Grande, P. 1989. Methylazoxymethanol acetate (MAM ac) effects on the ontogenesis of the mouse neocortex. *J. Hirnforsch.* **30**: 699–705. [Medline]
5. Corona-Rivera, J. R., Rea-Rosas, A., Santana-Ramírez, A., Acosta-León, J., Hernández-Rocha, J. and Miguel-Jiménez, K. 2010. Holoprosencephaly and genitourinary anomalies in fetal methotrexate syndrome. *Am. J. Med. Genet. A.* **152A**: 1741–1746. [Medline] [CrossRef]
6. Craciunescu, C. N., Brown, E. C., Mar, M. H., Albright, C. D., Nadeau, M. R. and Zeisel, S. H. 2004. Folic acid deficiency during late gestation decreases progenitor cell proliferation and increases apoptosis in fetal mouse brain. *J. Nutr.* **134**: 162–166. [Medline]
7. Craciunescu, C. N., Johnson, A. R. and Zeisel, S. H. 2010. Dietary choline reverses some, but not all, effects of folate deficiency on neurogenesis and apoptosis in fetal mouse brain. *J. Nutr.* **140**: 1162–1166. [Medline] [CrossRef]
8. Cuellar, M. L. and Espinoza, L. R. 1997. Methotrexate use in psoriasis and psoriatic arthritis. *Rheum. Dis. Clin. North Am.* **23**: 797–809. [Medline] [CrossRef]
9. DeLoia, J. A., Stewart-Akers, A. M. and Creinin, M. D. 1998. Effects of methotrexate on trophoblast proliferation and local immune responses. *Hum. Reprod.* **13**: 1063–1069. [Medline] [CrossRef]
10. Denaxa, M., Chan, C. H., Schachner, M., Parnavelas, J. G. and Karagogeos, D. 2001. The adhesion molecule TAG-1 mediates the migration of cortical interneurons from the ganglionic eminence along the corticofugal fiber system. *Development* **128**: 4635–4644. [Medline]
11. Eichholzer, M., Tönz, O. and Zimmermann, R. 2006. Folic acid: a public-health challenge. *Lancet* **367**: 1352–1361. [Medline] [CrossRef]
12. Ferguson, S. A., Berry, K. J., Hansen, D. K., Wall, K. S., White, G. and Antony, A. C. 2005. Behavioral effects of prenatal folate deficiency in mice. *Birth Defects Res. A Clin. Mol. Teratol.* **73**: 249–252. [Medline] [CrossRef]
13. Furukawa, S., Abe, M., Usuda, K. and Ogawa, I. 2004. Indole-3-acetic acid induces microencephaly in rat fetuses. *Toxicol. Pathol.* **32**: 659–667. [Medline] [CrossRef]
14. Furukawa, S., Usuda, K., Abe, M., Hayashi, S. and Ogawa, I. 2007. Indole-3-acetic acid induces microencephaly in mouse fetuses. *Exp. Toxicol. Pathol.* **59**: 43–52. [Medline] [CrossRef]
15. Gaglioti, P., Oberto, M. and Todros, T. 2009. The significance of fetal ventriculomegaly: etiology, short- and long-term outcomes. *Prenat. Diagn.* **29**: 381–388. [Medline] [CrossRef]
16. Garcia-Minaur, S. and Botella, M. P. 2000. Further case of aminopterin syndrome sine aminopterin in a Spanish child. *Am. J. Med. Genet.* **95**: 320–324. [Medline] [CrossRef]
17. Genestier, L., Paillot, R., Quemeneur, L., Izeradjene, K. and Revillard, J. P. 2000. Mechanisms of action of methotrexate. *Immunopharmacology* **47**: 247–257. [Medline] [CrossRef]
18. Gressens, P., Kosofsky, B. E. and Evrard, P. 1992. Cocaine-induced disturbances of corticogenesis in the developing murine brain. *Neurosci. Lett.* **140**: 113–116. [Medline] [CrossRef]
19. Guerrini, R., Sicca, F. and Parmeggiani, L. 2003. Epilepsy and malformations of the cerebral cortex. *Epileptic Disord.* **5** Suppl 2: S9–S26. [Medline]
20. Herman, S., Zurgil, N. and Deutsch, M. 2005. Low dose metho-

trexate induces apoptosis with reactive oxygen species involvement in T lymphocytic cell lines to a greater extent than in monocytic lines. *Inflamm. Res.* **54**: 273–280. [Medline] [CrossRef]
21. Juric-Sekhar, G., Kapur, R. P., Glass, I. A., Murray, M. L., Parnell, S. E. and Hevner, R. F. 2011. Neuronal migration disorders in microcephalic osteodysplastic primordial dwarfism type I/III. *Acta Neuropathol.* **121**: 545–554. [Medline] [CrossRef]
22. Kerjan, G. and Gleeson, J. G. 2007. Genetic mechanisms underlying abnormal neuronal migration in classical lissencephaly. *Trends Genet.* **23**: 623–630. [Medline] [CrossRef]
23. Kim, G. W. and Chan, P. H. 2001. Oxidative stress and neuronal DNA fragmentation mediate age-dependent vulnerability to the mitochondrial toxin, 3-nitropropionic acid, in the mouse striatum. *Neurobiol. Dis.* **8**: 114–126. [Medline] [CrossRef]
24. Kobayashi, K., Terada, C. and Tsukamoto, I. 2002. Methotrexate-induced apoptosis in hepatocytes after partial hepatectomy. *Eur. J. Pharmacol.* **438**: 19–24. [Medline] [CrossRef]
25. Komada, M., Fujiyama, F., Yamada, S., Shiota, K. and Nagao, T. 2010. Methylnitrosourea induces neural progenitor cell apoptosis and microcephaly in mouse embryos. *Birth Defects Res. B Dev. Reprod. Toxicol.* **89**: 213–222. [Medline]
26. Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V. and Kastan, M. B. 1992. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl. Acad. Sci. U.S.A.* **89**: 7491–7495. [Medline] [CrossRef]
27. Kuwagata, M., Ogawa, T., Shioda, S. and Nagata, T. 2009. Observation of fetal brain in a rat valproate-induced autism model: a developmental neurotoxicity study. *Int. J. Dev. Neurosci.* **27**: 399–405. [Medline] [CrossRef]
28. Lavdas, A. A., Grigoriou, M., Pachnis, V. and Parnavelas, J. G. 1999. The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. *J. Neurosci.* **19**: 7881–7888. [Medline]
29. Lidow, M. S. 1995. Prenatal cocaine exposure adversely affects development of the primate cerebral cortex. *Synapse* **21**: 332–341. [Medline] [CrossRef]
30. Liljeholm, M. and O'Doherty, J. P. 2012. Contributions of the striatum to learning, motivation, and performance: an associative account. *Trends Cogn. Sci. (Regul. Ed.)* **16**: 467–475. [Medline] [CrossRef]
31. Loeb, K. R. and Loeb, L. A. 2000. Significance of multiple mutations in cancer. *Carcinogenesis* **21**: 379–385. [Medline] [CrossRef]
32. Marchal, G., Andermann, F., Tampieri, D., Robitaille, Y., Melanson, D., Sinclair, B., Olivier, A., Silver, K. and Langevin, P. 1989. Generalized cortical dysplasia manifested by diffusely thick cerebral cortex. *Arch. Neurol.* **46**: 430–434. [Medline] [CrossRef]
33. McAllister, J. P. 2nd. 2012. Pathophysiology of congenital and neonatal hydrocephalus. *Semin. Fetal Neonatal Med.* **17**: 285–294. [Medline] [CrossRef]
34. McKechnie, L., Vasudevan, C. and Levene, M. 2012. Neonatal outcome of congenital ventriculomegaly. *Semin. Fetal Neonatal Med.* **17**: 301–307. [Medline] [CrossRef]
35. Miller, M. W. 1986. Effects of alcohol on the generation and migration of cerebral cortical neurons. *Science* **233**: 1308–1311. [Medline] [CrossRef]
36. Müller, M., Strand, S., Hug, H., Heinemann, E. M., Walczak, H., Hofmann, W. J., Stremmel, W., Krammer, P. H. and Galle, P. R. 1997. Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. *J. Clin. Invest.* **99**: 403–413. [Medline] [CrossRef]
37. Ogawa, T., Kuwagata, M., Muneoka, K. T. and Shioda, S. 2005. Neuropathological examination of fetal rat brain in the 5-bromo-2′-deoxyuridine-induced neurodevelopmental disorder model. *Congenit. Anom. (Kyoto)* **45**: 14–20. [Medline] [CrossRef]
38. O'Leary, D. D. and Koester, S. E. 1993. Development of projection neuron types, axon pathways, and patterned connections of the mammalian cortex. *Neuron* **10**: 991–1006. [Medline] [CrossRef]
39. Raedler, E., Raedler, A. and Feldhaus, S. 1980. Dynamical aspects of neocortical histogenesis in the rat. *Anat. Embryol. (Berl.)* **158**: 253–269. [Medline] [CrossRef]
40. Reynolds, E. 2006. Vitamin B12, folic acid, and the nervous system. *Lancet Neurol.* **5**: 949–960. [Medline] [CrossRef]
41. Sato, S., Gobbel, G. T., Honkaniemi, J., Li, Y., Kondo, T., Murakami, K., Sato, M., Copin, J. C. and Chan, P. H. 1997. Apoptosis in the striatum of rats following intraperitoneal injection of 3-nitropropionic acid. *Brain Res.* **745**: 343–347. [Medline] [CrossRef]
42. Scimeca, J. M. and Badre, D. 2012. Striatal contributions to declarative memory retrieval. *Neuron* **75**: 380–392. [Medline] [CrossRef]
43. Seidahmed, M. Z., Shaheed, M. M., Abdulbasit, O. B., Al Dohami, H., Babiker, M., Abdullah, M. A. and Abomelha, A. M. 2006. A case of methotrexate embryopathy with holoprosencephaly, expanding the phenotype. *Birth Defects Res. A Clin. Mol. Teratol.* **76**: 138–142. [Medline] [CrossRef]
44. Senuma, M., Mori, C., Ogawa, T. and Kuwagata, M. 2014. Prenatal sodium arsenite affects early development of serotonergic neurons in the fetal rat brain. *Int. J. Dev. Neurosci.* **38**: 204–212. [Medline] [CrossRef]
45. Sidman, R. L. and Rakic, P. 1973. Neuronal migration, with special reference to developing human brain: a review. *Brain Res.* **62**: 1–35. [Medline] [CrossRef]
46. Spurlock, C. F. 3rd., Tossberg, J. T., Fuchs, H. A., Olsen, N. J. and Aune, T. M. 2012. Methotrexate increases expression of cell cycle checkpoint genes via JNK activation. *Arthritis Rheum.* **64**: 1780–1789. [Medline] [CrossRef]
47. Strickland, K. C., Krupenko, N. I. and Krupenko, S. A. 2013. Molecular mechanisms underlying the potentially adverse effects of folate. *Clin. Chem. Lab. Med.* **51**: 607–616. [Medline] [CrossRef]
48. Sugiyama, A., Sun, J., Ueda, K., Furukawa, S. and Takeuchi, T. 2015. Effect of methotrexate on cerebellar development in infant rats. *J. Vet. Med. Sci.* **77**: 789–797. [Medline] [CrossRef]
49. Sun, J., Sugiyama, A., Inoue, S., Takeuchi, T. and Furukawa, S. 2014. Effect of methotrexate on neuroepithelium in the rat fetal brain. *J. Vet. Med. Sci.* **76**: 347–354. [Medline] [CrossRef]
50. Sun, X. Z., Takahashi, S., Cui, C., Zhang, R., Sakata-Haga, H., Sawada, K. and Fukui, Y. 2002. Normal and abnormal neuronal migration in the developing cerebral cortex. *J. Med. Invest.* **49**: 97–110. [Medline]
51. Sun, X. Z., Takahashi, S., Fukui, Y., Hisano, S., Kuboda, Y., Sato, H. and Inouye, M. 1999. Different patterns of abnormal neuronal migration in the cerebral cortex of mice prenatally exposed to irradiation. *Brain Res. Dev. Brain Res.* **114**: 99–108. [Medline] [CrossRef]
52. Twining, P., Jaspan, T. and Zuccollo, J. 1994. The outcome of fetal ventriculomegaly. *Br. J. Radiol.* **67**: 26–31. [Medline] [CrossRef]
53. Wani, N. A., Hamid, A. and Kaur, J. 2008. Folate status in various pathophysiological conditions. *IUBMB Life* **60**: 834–842. [Medline] [CrossRef]
54. Wessels, J. A., Huizinga, T. W. and Guchelaar, H. J. 2008. Recent insights in the pharmacological actions of methotrexate in the treatment of rheumatoid arthritis. *Rheumatology (Oxford)* **47**: 249–255. [Medline] [CrossRef]
55. Yang, Z. J., Wang, Y. W., Li, C. L., Ma, L. Q. and Zhao, X. 2015. Pre-treatment with a Xingnaojing preparation ameliorates sevoflurane-induced neuroapoptosis in the infant rat striatum. *Mol. Med. Rep.* **11**: 1615–1622. [Medline]