FULL PAPER *Pathology*

Effect of methotrexate on rostral migratory stream in newborn rats

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(Received 8 May 2015/Accepted 20 June 2015/Published online in J-STAGE 2 July 2015)

ABSTRACT. Two-day-old rats were treated with subcutaneous injections of methotrexate (MTX) 5 mg/kg and 150 mg/kg, and their rostral migratory streams (RMS) were examined time-dependently. MTX treatment increased pyknotic and TUNEL-positive cells and decreased mitotic and phospho-Histone H3-positive cells at almost all time points in the vertical arm, elbow and horizontal arm regions of the RMS. There were more TUNEL-positive cells ratio in the MTX 150 mg/kg group than in the MTX 5 mg/kg group. Treatment with MTX 150 mg/kg decreased the cellularity in the vertical arm region on Postnatal day (PD) 4, but that with the MTX 5 mg/kg did not. TUNEL-positive cells ratio was the highest in the vertical arm region, followed by elbow and horizontal regions in both MTX-treated groups. TUNEL-positive cells ratio in the vertical arm and elbow regions reached their peaks on PD 4 in both MTX-treated groups, and both MTX-treatments significantly decreased Phospho-Histone H3-positive cells ratio on PDs 2.5 and 3 in the vertical arm, elbow and horizontal arm regions. The phospho-Histone H3-positive cells ratio in the vertical arm region recovered on PD4 in the MTX 150 mg/kg group. These findings suggested that RMS required a great amount of folic acid on PD 2 and that the folic acid-requirement differed depending on the anatomical region of the RMS. To our knowledge, this is the first report demonstrating the effect of MTX on the RMS and the necessity of the folic acid metabolism on RMS development in newborn rats.

KEY WORDS: methotrexate, newborn, pyknosis, rat, rostral migratory stream

doi: 10.1292/jvms.15-0280; *J. Vet. Med. Sci.* 77(12): 1565–1571, 2015

Throughout life, the anterior part of the subventricular zone (SVZ) of rodents contains a prolific source of neuronal progenitor cells that retains the capacity to concurrently generate neurons [24]. The neuroblasts from the anterior SVZ migrate along a defined pathway, the rostral migratory stream (RMS), into the olfactory bulb and differentiate into granular and periglomerular interneurons [32]. It is well-known that neuroblasts of the SVZ maintain the ability to divide during their migration within the RMS [19]. The RMS is divided into 3 anatomical regions: (1) the vertical arm which extends from the SVZ and includes the descending region underlying the white matter of the corpus callosum; (2) the elbow, composed of the rostral curve toward the olfactory bulb at the base of the vertical arm; and (3) the horizontal arm, which is the final rostral extension into the olfactory bulb [20]. Martoncíková *et al*. [18] demonstrated that there were quantitative differences in the extent of cell proliferation within RMS during the first postnatal month (Postnatal day (PD) 0–28) in rats. They showed that the number of proliferating cells in the vertical arm, elbow and horizontal arm regions on PD 0 and PD 3 was higher than at other time points [18].

There have been several reports demonstrating the adverse effect of external factors on RMS in infant and adult rats and mice [5, 9, 21, 23, 25]. Maternal separation induced an inhibition of cell proliferative activity and increased the number of dying cells in the RMS of infant rats [25]. Exposure to single whole-body gamma irradiation with the dose of 3 Gy inhibited cell proliferative activity in the RMS in the brain of adult rats [5], and exposure of infant rats to electromagnetic fields for 8 hr/day for 3 days to infant rats of PD 7 induced a decrease in BrdU-positive cells in the RMS [23]. Moreover, peripherally injected lipopolysaccharide induced apoptotic cell death in the RMS of young adult mice [21], and a single acute injection of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine induced apoptosis in the RMS of the adult C57BL/6 mice [9].

A folic acid metabolism antagonist, methotrexate (MTX), inhibits the reduction of dihydrofolate to tetrahydrofolate, a metabolically active agent [12, 17, 31]. This serves to prevent *de novo* pyrimidine and purine syntheses, required for DNA and RNA syntheses, consequently inhibits cell proliferation and induces apoptosis [12, 17, 31]. There have been several reports demonstrating the effect of postnatal MTX exposure on the central nervous system. MTX treatment on PD 6 induced apoptosis and decreased cell proliferative activity of external granular cells, resulting in cerebellar hypoplasia in rats [29]. MTX also induced apoptosis and reduced the number of Ki-67-positive cells and doublecortin (immature progenitor neuron marker)-positive cells in the adult mouse hippocampus [33]. Although MTX decreased the number

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of Ki-67-positive cells in the adult rat hippocampus [26, 27], there have been no reports to date describing the effect of postnatal exposure to MTX on the RMS development of newborn animals. In the present study, we carried out a sequential histopathological examination of the RMS of newborn rats following MTX administration on PD 2, to elucidate just what effect MTX has on newborn rat RMS and to determine the significance of folic acid metabolism on the RMS development of newborn rats.

MATERIALS AND METHODS

Animals: Newborn male rats were obtained at Tottori University by mating specific pathogen-free male and female rats of the Wistar Imamichi strain, purchased from the Institute of Animal Reproduction (Kasumigaura, Japan). One dam with 5 male infants was housed together in cages (W335 × D380 × H175 mm) with bedding (CLEA Japan, Inc., Tokyo, Japan). Because gender differences in MTX-induced pathological changes in the RMS were not observed in the preliminary study, male rats were used in the main study.

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 were 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 75 two-day-old male rats were divided into three groups as follows: (1) saline-treated control rats (n=25), (2) MTX 5 mg/kg-treated rats (n=25) and (3) MTX 150 mg/kg-treated rats (n=25). MTX (Pfizer Japan Inc., Tokyo, Japan) was dissolved in saline. The two-day-old rats received subcutaneous injection with MTX (5 mg/kg or 150 mg/kg body weight) or saline (the control).

Two-day-old male rats were treated with 5 (n=25) or 150 mg/kg (n=25) of MTX or saline (n=25), and their brains were sampled on PDs 2.5, 3, 4, 5 and 6 (n=5 per group) to examine the time-course histopathological changes in the RMS after euthanasia by overdose administration of pentobarbiturate (100 mg/kg, i.p.). The specific timing of MTX administration we used was selected, because component cells of the RMS in the present period showed high cell proliferative activity in the previous study [18]. In our preliminary study, MTX 0.5 and 1 mg/kg treatment on PD 2 induced no histopathological changes, but MTX 5 mg/kg treatment on PD 2 induced pyknotic changes in the RMS of newborn rats. Our preliminary study revealed that MTX 150 mg/kg was the minimal dose that decreased cellularity in the vertical arm region on PD 4. The dose of MTX in the present study was designated as 5 mg/kg and 150 mg/kg, based on the results of our preliminary study.

Histopathological examination: All brain samples were fixed in 10% neutral buffered formalin, embedded in paraffin, then sectioned and stained with hematoxylin and eosin (HE).

DNA-fragmented external granular cells were detected by terminal deoxynucleotidyl-transferase (TdT)-mediated deoxyuridine triphosphate-digoxigenin (dUTP) nick-end labeling (TUNEL) using an *in situ* apoptosis detection kit (Trevigen, Inc., Gaithersburg, MD, U.S.A.). TUNEL-positive cells in the RMS were counted from over 500 cells by light microscopy for each newborn animal, and the TUNEL index was calculated as the percentage of TUNEL-positive cells from all of the RMS component cells counted.

Immunohistochemical staining was performed by a labeled polymer method using a detection reagent, 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) and microwaved for 15 min. Endogenous peroxidase activity was quenched by immersing the sections in 3% hydrogen peroxide in methanol for 15 min. 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 were treated with Histofine Simple Stain MAX-PO (R) (Nichirei) 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 Phospho-Histone H3-positive cells in the RMS were counted from over 500 external granule cells of each newborn animal by light microscopy, and the Phospho-Histone H3-index was calculated as the percentage of Phospho-Histone H3-positive cells out of all RMS-component cells counted.

Statistical analysis: All data are expressed as means ± standard error (SE) in each group. The results in each group were compared by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or Dunnett's multiple comparison test with statistical software ("Excel Toukei 2015", SSRI Co., Ltd., Tokyo, Japan). $P<0.05$ or $P<0.01$ was considered to be statistically significant.

RESULTS

Newborn rats neither in the control nor the two MTX-treated groups showed any clinical signs during the experimental period.

Both MTX 5 and 150 mg/kg treatment increased pyknotic- and TUNEL-positive cells (Figs. 1 and 2) and decreased mitotic and Phospho-Histone H3-positive cells (Figs. 3 and 4) at almost all time points in the vertical arm, elbow and horizontal arm regions of the RMS. In the MTX 150 mg/kg group, there were many pyknotic- and TUNEL-positive cells in the vertical arm region on PD 4, and RMS-component cells were very sparse in the vertical arm region on PD 4 (Fig. 1). In the MTX 150 mg/kg group, sparsity of RMS-component cells was not observed in all anatomical regions of the RMS and olfactory bulb at any time points, except for the vertical arm region on PD 4 (Fig. 1). MTX 5 mg/kg treatment did not induce a sparsity in cellularity of RMS-component cells in any anatomical regions of RMS and olfactory bulb at any time points (Fig. 1). TUNEL index in the MTX 150 mg/kg group was higher than that in the

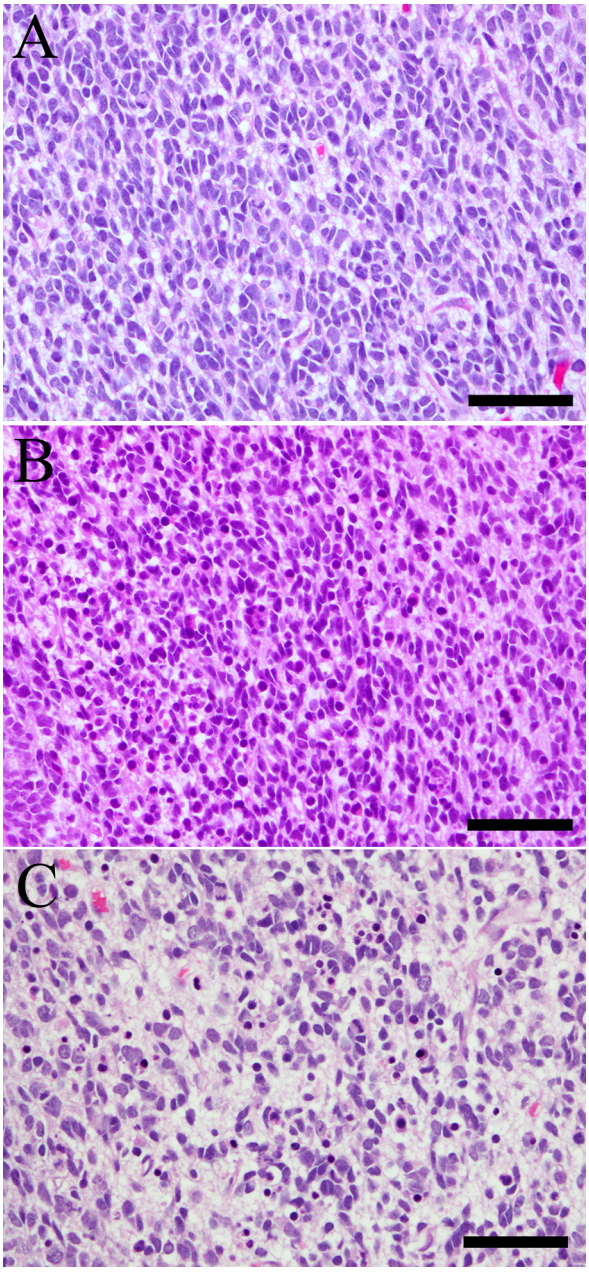

Fig. 1. Vertical arm region of RMS on PD 4 in MTX-treated infant rats. (A) Control group, (B) MTX 5 mg/kg-treated group, (C) MTX 150 mg/kg-treated group. Bar=50 μm. (B) In MTX 5 mg/kg group, pyknotic cells were scattered in vertical arm region. Sparsity of RMS-component cells was not observed. (C) In MTX 150 mg/kg group, there were many pyknotic cells in vertical arm region. RMS-component cells of vertical arm region were very sparse.

MTX 5 mg/kg group at almost all time points in the vertical arm, elbow and horizontal arm regions (Fig. 5). TUNEL-index in the vertical arm, elbow and horizontal arm regions reached their peaks on PD 4 in both MTX 150 mg/kg- and 5 mg/kg-treated groups (Fig. 5). Also, TUNEL-index in the

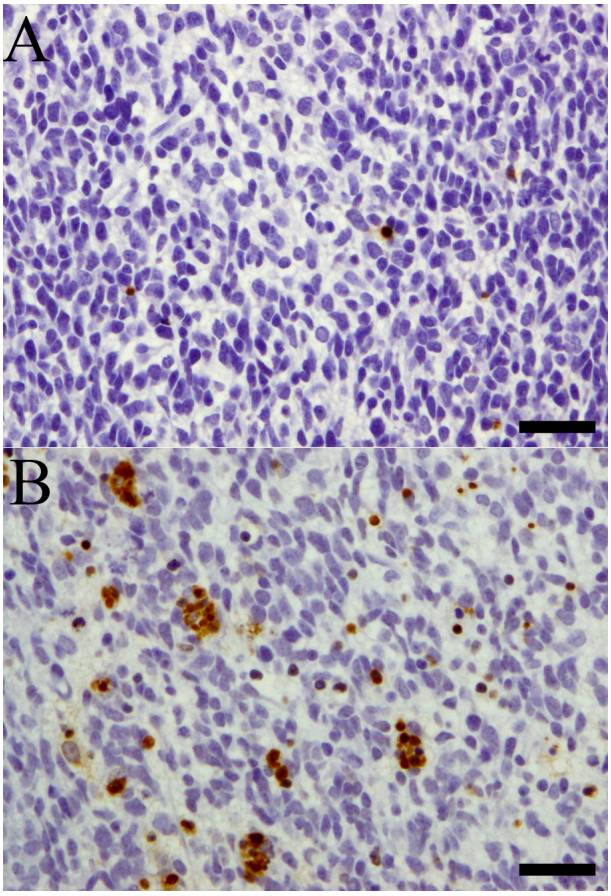

Fig. 2. TUNEL-positive cells in vertical arm of RMS on PD 4 in MTX-treated infant rats. (A) Control group, (B) MTX 150 mg/kg-treated group. Bar=30 μm.

vertical arm region was the highest, and the elbow and the horizontal arm regions followed it at all time points in both MTX-treated groups (Fig. 6). Both MTX 5 and 150 mg/kg treatment significantly decreased the Phospho-Histone H3-positive cell index on PDs 2.5 and 3 in the vertical arm, elbow and horizontal arm regions (Fig. 7). On PD 4 in the MTX 150 mg/kg group, Phospho-Histone H3-index in the vertical arm region recovered and showed a tendency to be higher than that in the control group (Fig. 7). On PD 5 in the MTX 5 mg/kg group, Phospho-Histone H3-index in the elbow region recovered and showed a tendency to be higher than that in the control group (Fig. 7). On PD 6, Phospho-Histone H3-index in the horizontal arm region in the MTX 5 mg/kg group was significantly higher than that in the control group (Fig. 7).

DISCUSSION

Several previous studies revealed that folate deficiency at postnatal periods induces brain developmental disorder in human infants and infant animals [2, 6]. One previous human study showed that there was a delay in the maturation pattern

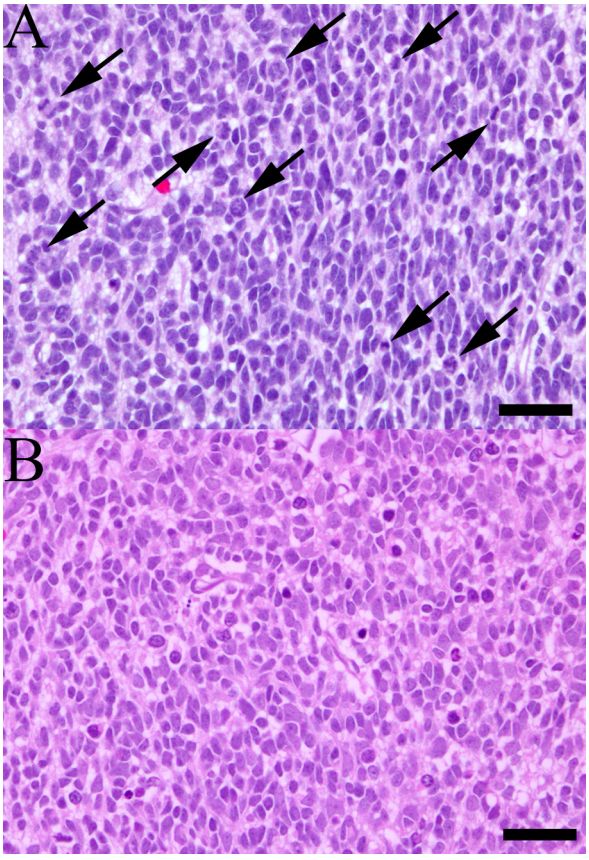

Fig. 3. Mitotic cells in vertical arm of RMS on PD 2.5 in MTX-treated infant rats. (A) Control group, (B) MTX 150 mg/kg -treated group. Bar=30 μm. Arrows show mitotic cells.

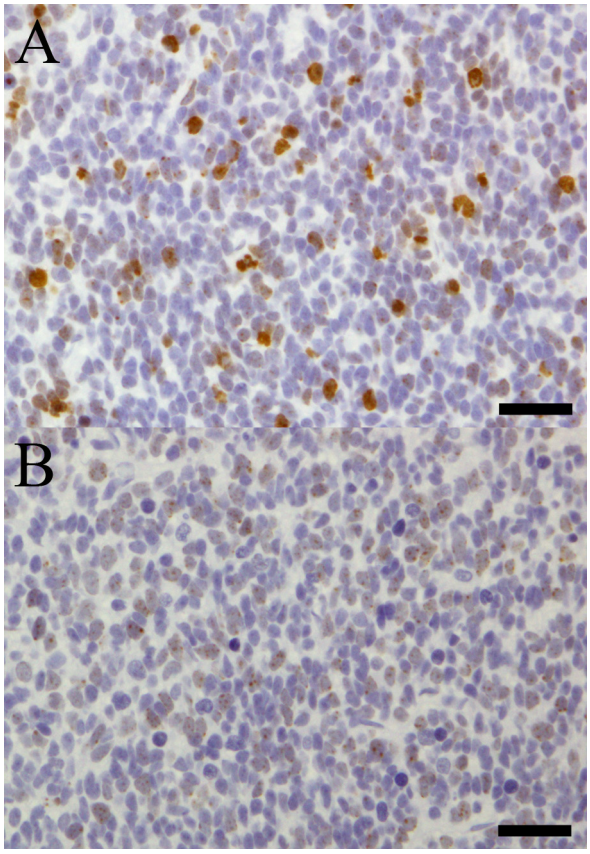

Fig. 4. Immunohistochemical expression of Phospho-Histone H3 in vertical arm of RMS on PD 2.5 in MTX-treated infant rats. (A) Control group, (B) MTX 150 mg/kg-treated group. Bar=30 μm.

of electroencephalogram in infants fed milk from mothers with low serum folate levels [2]. Berrocal-Zaragoza *et al*. [6] reported that folate deficiency in rat pups during weaning caused learning and memory deficits. However, there have been no reports elucidating an association between folate deficiency in postnatal periods with the RMS development in newborn animals.

In the present study, MTX at 5 and 150 mg/kg on PD 2 induced apoptosis and inhibited cell proliferation in RMS. MTX 150 mg/kg markedly decreased the cellularity in the vertical arm region on PD 4. These findings revealed that RMS-component cells in the present period greatly required folic acid. Folates have a central role in one-carbon metabolism and are critical coenzymes for nucleotide synthesis in cell proliferation [7]. Therefore, the need for folic acid is closely associated with cell proliferative activity. A previous study revealed that there was a very large number of proliferating cells in RMS during PD 0 and PD 3, and in the present period, RMS-component cells showed very high cell proliferative activity [18].

A previous study demonstrated that folate deficiency induced apoptosis and cell proliferation inhibition of neural stem cells by increasing homocysteine *in vitro* [34]. Another study showed that dietary folic acid deficiency reduced the number of proliferating neuronal progenitor cells in the dentate gyrus of the hippocampus and increased blood homocysteine levels in adult mice [16]. Homocysteine is a neurotoxic amino acid that induces neurotoxicity by multiple routes, such as increasing cytosolic calcium and oxidative stress, decreasing endogenous antioxidants, depleting cellular methylation reactions, inducing mitochondrial and DNA damage, and depleting energy reserves and overactivation of kinases [10, 11, 14, 15, 22, 30]. Apoptosis and cell proliferation inhibition of RMS-component cells induced by MTX in the present study may also be associated with an increase of homocysteine.

Raceková *et al*. [25] showed that maternal separation decreased the number of proliferating cells in RMS of infant rats. This decrease was more prominent in the vertical arm and elbow regions than the horizontal arm region [25]. The density of proliferating cells decreased following a caudorostral gradient, with the maximum around the lateral ventricle and minimum in the subependymal layer of the olfactory bulb [1]. Raceková *et al*. [25] hypothesized that the regional differences in response by the anatomical regions of the RMS to maternal separation could be attributed to

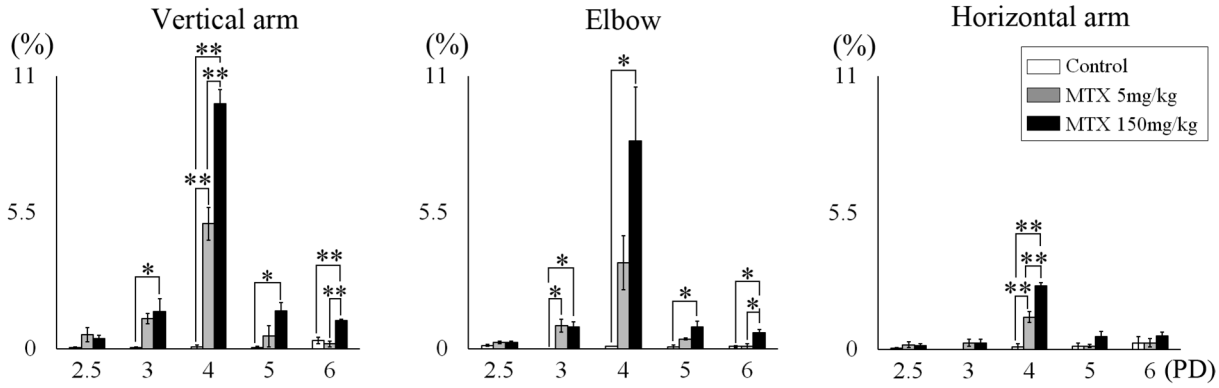

Fig. 5. Time course changes in TUNEL index of the component cells of RMS: the comparison between saline-treatment, MTX 5 mg/kg-treatment and MTX 150 mg/kg-treatment. Values are expressed as means ± SE. *, **: Significant difference at $P<0.05$, $P<0.01$ by one-way analysis of variance followed by Tukey's multiple comparison test.

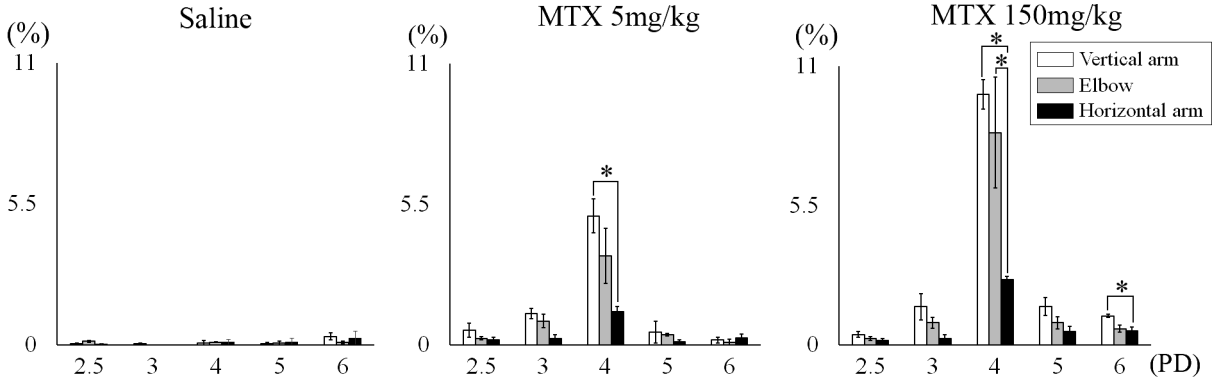

Fig. 6. Time course changes in TUNEL index of the component cells of RMS: the comparison between vertical arm region, elbow region and horizontal arm region. Values are expressed as means ± SE. *: Significant difference at $P<0.05$ compared by one-way analysis of variance followed by Tukey's multiple comparison test.

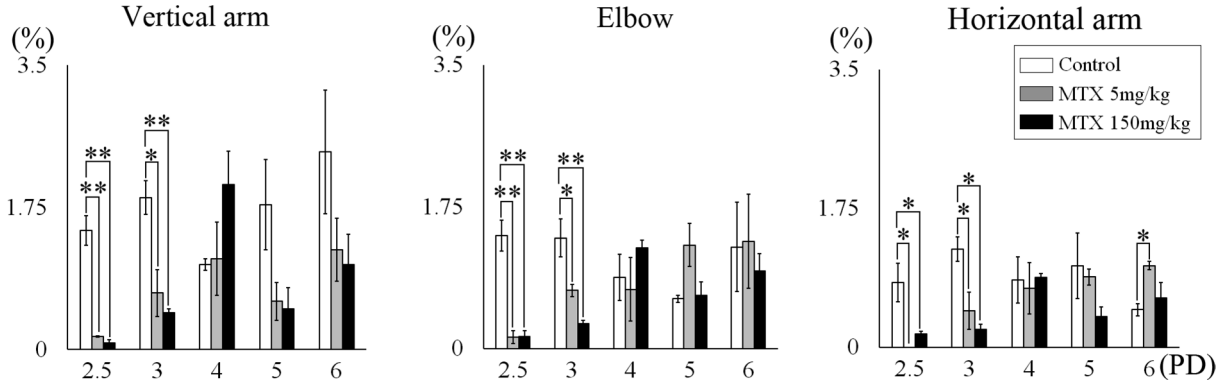

Fig. 7. Time course changes in Phospho-Histone H3-indices in the component cells in RMS. Values are expressed as means ± SE. *, **: Significantly different at $P<0.05$, $P<0.01$ by one-way analysis of variance followed by Tukey's multiple comparison test.

the differences in the cell proliferative activities of RMS-component cells. In the present study, the vertical arm region was most strongly affected by MTX, followed by elbow and horizontal arm regions. The difference in sensitivity to MTX may also be attributed to differences in cell proliferative activity in RMS.

In the present study, both MTX treatment significantly decreased the Phospho-Histone H3-positive cell index on

PD 2.5 and 3 in the vertical arm, elbow and horizontal arm regions. On PD 4 in the MTX 150 mg/kg group, Phospho-Histone H3-index in the vertical arm region recovered and showed a tendency to be higher than that in the control group. On PD 5 in the MTX 5 mg/kg group, Phospho-Histone H3-index in the elbow region recovered and showed a tendency to be higher than that in the control group. On PD 6, Phospho-Histone H3-index in the horizontal arm region in MTX 5 mg/kg group was significantly higher than that in the control group. Bálentová *et al*. [4] demonstrated that a significant increase in cell proliferation was observed in the vertical arm and elbow regions of adult rats 25 days after irradiation with a single sublethal whole-body dose of 3 Gy by gamma rays. Shinohara *et al*. [28] showed that recovery of cell proliferation in other germinative zones, such as subependyma of the brain in young adult rats, was brought about with a single dose of 3 Gy, as was observed from 50 hr after irradiation. The proliferative response that follows cellular depletion by apoptosis may result from the supply of cells from neural stem cells [28], which can be stimulated by injury to compensate for the loss of the damaged neurons in adult brain [13]. It is considered that in the present study, proliferation of Phospho-Histone H3-index that follows its decrease is also a compensatory change for the loss of the damaged neuroblasts.

Bálentová *et al*. [3] showed that whole-body irradiation with fractionated doses of gamma rays resulted in strong enhancement of astrocytes in the elbow and horizontal regions in the RMS of adult rats. Fukushima *et al*. [8] demonstrated that complete resection of RMS in adult rats significantly reduced proliferating cells in the olfactory bulbar granule cell layer. However, complete resection of RMS decreased the rate of neurogenesis, and the rate of gliogenesis tended to increase [8]. This finding suggested that the migrating neural stem cells of periventricular origin were associated with both neurogenesis and gliogenesis, while the local stem cells of intrabulbar origin were predominantly associated with gliogenesis in the olfactory bulb [8]. In the present study, MTX treatment markedly decreased the cellularity of the vertical arm region, but not the elbow, horizontal arm regions and olfactory bulb. This result may be attributed to compensatory gliogenesis in the elbow and horizontal arm regions and olfactory bulb.

In conclusion, MTX administration at 5 and 150 mg/kg on PD 2 induced apoptosis of component cells in the RMS and inhibited their proliferation. MTX 150 mg/kg treatment decreased cellularity of the vertical arm region, but not MTX 5 mg/kg. MTX treatment affected the vertical arm region most strongly, followed by the elbow and horizontal arm regions. These findings suggested that RMS-component-cells on PD 2 required much folic acid and that the folic acid-requirement was different depending on the anatomical regions of RMS. This is, to our knowledge, the first research report clarifying the significance of the folic acid metabolism on RMS development in newborn rats.

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