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Short Communication

Histopathologic effect of in ovo exposure to methotrexate at early embryonic stage on optic tectum of Japanese quail (*Coturnix japonica*)

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Abstract: The optic tectum of Japanese quail embryos with in ovo exposure to methotrexate 100 ng/g egg on embryonic day 4 was examined from 3 to 24 hour after treatment. At 9 hour after methotrexate exposure, several apoptotic neuroepithelial cells appeared in the ventricular zone of the optic tectum; these increased in number and were diffusely distributed throughout all layers of the ventricular zone of the optic tectum at 12 hour. At 24 hour, neuroepithelial cells in the ventricular zone of the optic tectum were eliminated and showed sparse cell density. Throughout the experimental period, proliferation of neuroepithelial cells in the ventricular zone of the optic tectum of methotrexate-treated embryos was inhibited. These results suggest that neuroepithelial cells in the ventricular zone of the optic tectum in Japanese quail embryos can be affected by folic acid antimetabolites, methotrexate, at an early embryonic stage. (DOI: 10.1293/tox.2022-0011; J Toxicol Pathol 2022; 35: 269–274)

Key words: apoptosis, cell proliferation inhibition, in ovo exposure, Japanese quail embryo, methotrexate, optic tectum

Methotrexate (MTX) is an antimetabolite that interferes with the metabolism of folic acid[1]. MTX binds dihydrofolate reductase with an affinity 1,000-fold greater than that of folate, and competitively inhibits conversion of dihydrofolate to tetrahydrofolate[1–3]. Tetrahydrofolate is essential for biosynthesis of thymidine and purine, which are needed for synthesis of DNA[1, 2]. Blockade of tetrahydrofolate synthesis by MTX leads to inhibition of cell proliferation[2, 4]. MTX administration during pregnancy induced anomalies of the central nervous system (CNS) in fetuses and infants of humans and experimental animals[2].

MTX is used clinically in the treatment of neoplastic diseases, such as acute lymphoblastic leukemia, and autoimmune and chronic inflammatory diseases, including rheumatoid arthritis, sarcoidosis and psoriasis[2–5]. Previous studies demonstrated that MTX was detected in the hospital effluents, the wastewater treatment plant influents and effluents and the surface water[6–11]. Therefore, there is a possibility that MTX contaminates the natural environment and exerts adverse effects on CNS development in the fetuses/embryos of vertebrate animals inhabiting such areas. Nevertheless, there are few reports examining the effect of MTX on CNS development in embryos of avian species. Only one research paper has demonstrated the effect of in ovo MTX exposure on the optic tectum of embryos in avian species[12]. This research showed that in ovo MTX exposure on embryonic day (ED) 3.5 induced a decrease in the weight of the optic tectum in chick embryos on ED 18[12]. However, histopathological examination of the optic tectum in embryos exposed to MTX was not performed and the histopathologic effect of MTX on the optic tectum has not been clarified[12]. For the purpose of elucidating the effect of MTX on the CNS development in avian species, we conducted the preliminary experiment using Japanese quail embryos. The results of our preliminary experiment showed that ovo exposure to MTX on ED 4 induced obvious histopathological changes in the optic tectum of Japanese quail embryos. Accordingly, the optic tectum was selected as a target of research in the present study. The specific timing of MTX exposure (ED 4) was selected in reference to a previous study, which demonstrated that neuroepithelial cells in the ventricular zone of the optic tectum show high cell proliferative activity during ED 1–4[13].

In the present study, we examined temporal histopathological changes in the optic tectum of Japanese quail (*Coturnix japonica*) embryos after in ovo exposure to MTX on ED 4. The purpose of the present study was to elucidate the histopathologic effect of MTX exposure at an early embryonic stage on the optic tectum of avian embryos.

Fertilized eggs of Japanese quail (NIES-L strain) were obtained from the Avian Bioscience Research Center, Graduate School of Bioagricultural Sciences, Nagoya University

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through the National Bio-Resource Project of the MEXT, Japan. Japanese quail demonstrates high egg production performance, and inbred strains of Japanese quail have been established[14, 15]. Additionally, in comparison to the chicken eggs, Japanese quail eggs are small, and their incubation period (embryonic development period) are short[14–16]. Therefore, the Japanese quails were selected as test animals in this study. The eggs were incubated at 39.5°C and 60% relative humidity and turned at 1 h intervals using an electric incubator (SHOWA FRANKI, Saitama, Japan). The present experiments were performed following the provisions approved by the Animal Research Committee of Okayama University of Science.

A total of 100 eggs were divided into 2 groups as follows: (1) saline-treated control eggs (n=50), and (2) MTX-treated eggs (n=50). MTX (Pfizer Japan Inc., Tokyo, Japan) was dissolved in saline. On day 4 of incubation (ED 4), MTX at 100 ng/g egg or saline (test solution: 2 μL/g egg) was injected into the egg yolk with a 25-gauge needle attached to a Hamilton syringe through a hole in the shell, which had been punched with a 25-gauge needle, at the blunt end of the egg. After injection, the shell was sealed with a sterilized patch (NICHIBAN Co., Ltd., Tokyo, Japan) and the eggs were returned to the incubator. Our preliminary study using Japanese quail embryos at 24 h following MTX exposure on ED 4 showed that exposure to MTX at 0.1 ng/g, 1.0 ng/g and 10 ng/g egg induced no significant histopathological changes in the ventricular zone of the optic tectum, whereas exposure to MTX at 100 ng/g egg caused stable histopathological changes. Based on the results of our preliminary study, the dose of MTX in the present study was designated as 100 ng/g egg. Embryo samples were collected at 3, 6, 9, 12 and 24 h after in ovo exposure to MTX or saline. At 3–12 h after treatment, the number of embryo samples in MTX or saline-treated group at each time point is ten. At 24 h after treatment, the number of embryo samples in saline-treated group was ten, whereas that in MTX-treated group was eight, because two embryos had died.

All embryos were fixed in Bouin's fluid overnight before being fixed again in 10% neutral buffered formalin, embedded in paraffin, cut into sagittal sections, and routinely stained with hematoxylin-eosin. The top part of the optic tectum was histopathologically analyzed in the present study (Fig. 1). The present part was selected as a representative one, because the histopathological changes were induced circumferentially in the optic tectum and there was no difference in their severity depending on the location.

Immunohistochemical staining was carried out using a labelled polymer method with the EnVision+ System (Catalog No. K4003, Dako Denmark A/S, Glostrup, Denmark). For antigen retrieval, tissue sections for the detection of cleaved caspase-3 were immersed in citrate buffer at pH 6.0 and autoclaved for 15 min at 121°C. Tissue sections for the detection of phospho-histone H3 were immersed in citrate buffer and microwaved for 15 min. Endogenous peroxidase activity in the sections was quenched by immersing the sections in 3% hydrogen peroxide in methanol for 15 min. The sections were incubated with a cleaved caspase-3 rabbit polyclonal antibody (Catalog No. 9661, 1:300 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight, or a phospho-histone H3 rabbit monoclonal antibody (Catalog No. ab32107, 1:1,500 dilution; Abcam, Cambridge, UK) for 30 min at room temperature. This phospho-histone H3 antibody detects histone H3 phosphorylated on both serine 10 and threonine 11. Sections were then treated using the EnVision+ System (Dako Denmark A/S) for 30 min at room temperature. After exposure to a 3,3′-diaminobenzidine solution containing hydrogen peroxide to facilitate a peroxidase color reaction, the sections were counterstained with Mayer's hematoxylin. All embryo samples were stained immunohistochemically for cleaved caspase-3 or phospho-histone H3. Using all embyo samples, the cleaved caspase-3-positive rate and the phospho-histone H3-positive rate were calculated as the percentages of cleaved caspase-3-positive cells and phospho-histone H3-positive cells, respectively, among the total number of neuroepithelial cells in the ventricular zone of the top part (Fig. 1) of the optic tectum using the histomorphometric analysis software (Olympus Corporation, Tokyo, Japan).

All values are expressed as the mean ± standard error. Comparisons of differences between the control and MTX-treated groups were analyzed using Excel-Toukei statistical software (SSRI Co., Ltd., Tokyo, Japan). The data from the two groups were analyzed using an *F*-test. When variances were homogenous, Student's t-test was performed. Welch's t-test was performed when variances were not homogeneous ($p<0.05$). P values less than 0.05 and 0.01 were considered

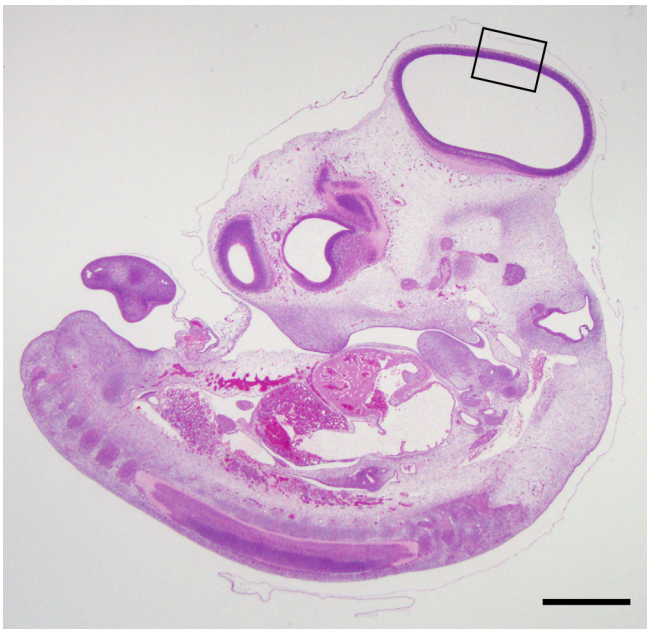

Fig. 1. Microscopic image of a sagittal section of a control Japanese quail embryo (3 h after saline exposure). The region enclosed by a line in the optic tectum was histopathologically analyzed in the present study. Bar=1 mm. Hematoxylin-eosin stain.

indicative of statistical significance.

In the control group, there were few pyknotic neuroepithelial cells in the ventricular zone of the optic tectum throughout the experimental period (Fig. 2a). At 3 and 6 h after MTX exposure, few pyknotic neuroepithelial cells were observed in the ventricular zone of the optic tectum (Fig. 2b and 2c). At 9 h after MTX treatment, several pyknotic neuroepithelial cells appeared in the ventricular zone of the optic tectum, and these increased in number and were diffusely distributed throughout all layers of the ventricular zone of the optic tectum at 12 h (Fig. 2d and 2e). Neuroepithelial cells in the ventricular zone of the optic tectum were eliminated and showed sparse cell density at 24 h (Fig. 2f). Many mitotic neuroepithelial cells were located along the ventricular surface of the optic tectum in control embryos, whereas they were rarely observed in the same region in MTX-treated embryos throughout the experimental period (Fig. 2a–f). There were few cleaved caspase-3-positive neuroepithelial cells in the ventricular zone of the optic tectum in control embryos throughout the experimental period (Fig. 3a and 3e). In the ventricular zone of the optic tectum of MTX-treated embryos, almost all pyknotic neuroepithelial cells were positive for cleaved caspase-3 (Fig. 3b). At 9, 12 and 24 h after MTX exposure, the cleaved caspse-3-positive rate in the ventricular zone of the optic tectum of MTX-treated embryos was significantly higher than that of

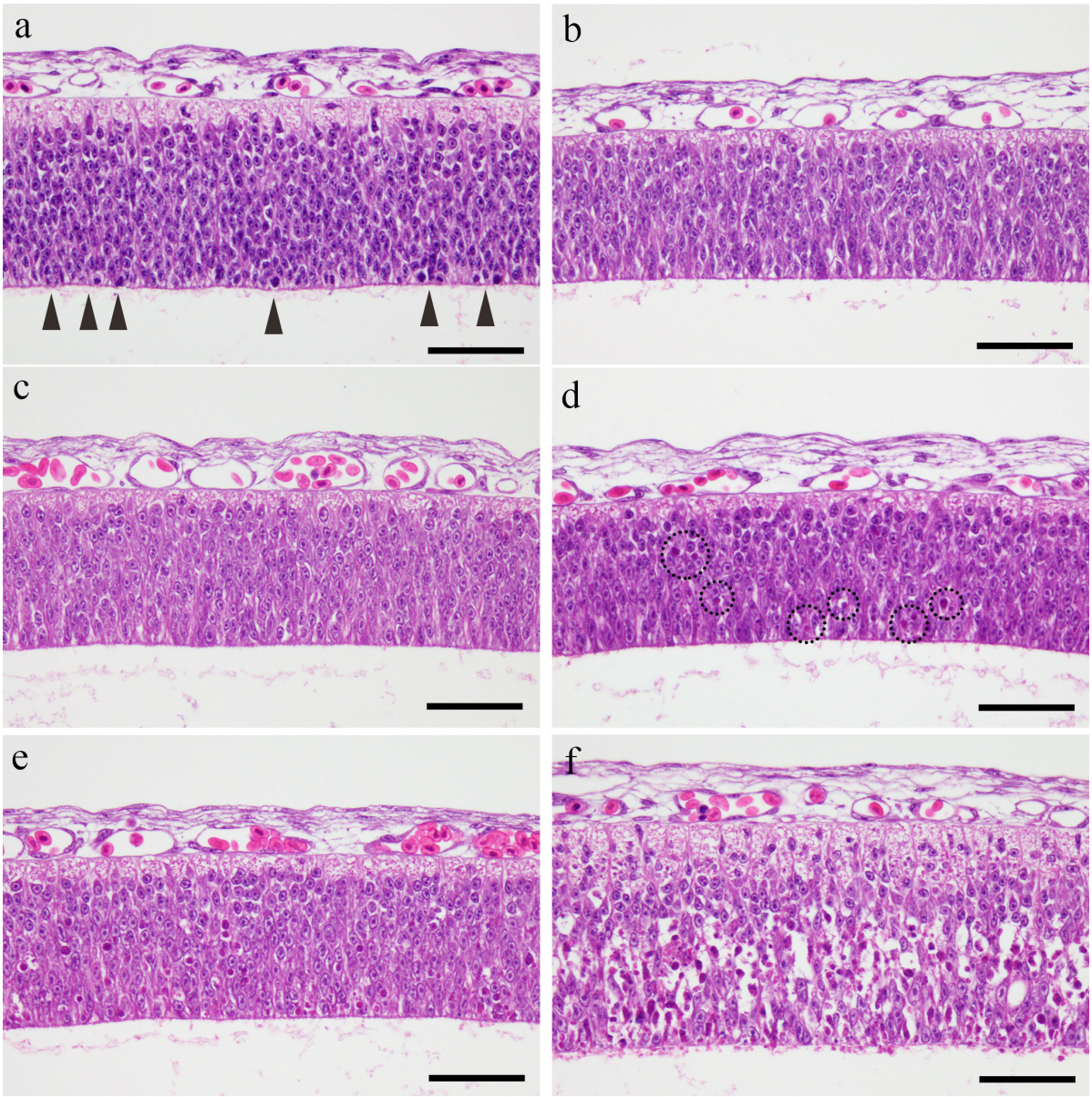

Fig. 2. Histopathological findings of the optic tectum in a control Japanese quail embryo at 3 h after saline treatment (a) and in MTX-treated embryos at 3 h (b), 6 h (c), 9 h (d), 12 h (e), 24 h (f) after treatment. Bar=50 µm. (a) Arrowheads indicate mitotic cells. (d) Pyknotic cells are surrounded by dotted lines. Hematoxylin-eosin stain.

the control embryos (Fig. 3e). Throughout the experimental period, many phospho-histone H3-positive neuroepithelial cells were localized along the ventricular surface of the optic tectum in the control embryos, while few were observed in the same region in MTX-treated embryos (Fig. 3c and 3d). Throughout the experimental period, the phospho-histone H3-positive rate in the ventricular zone of the optic tectum of MTX-treated embryos was significantly lower than that of the control (Fig. 3f).

The results of the present study revealed that in ovo

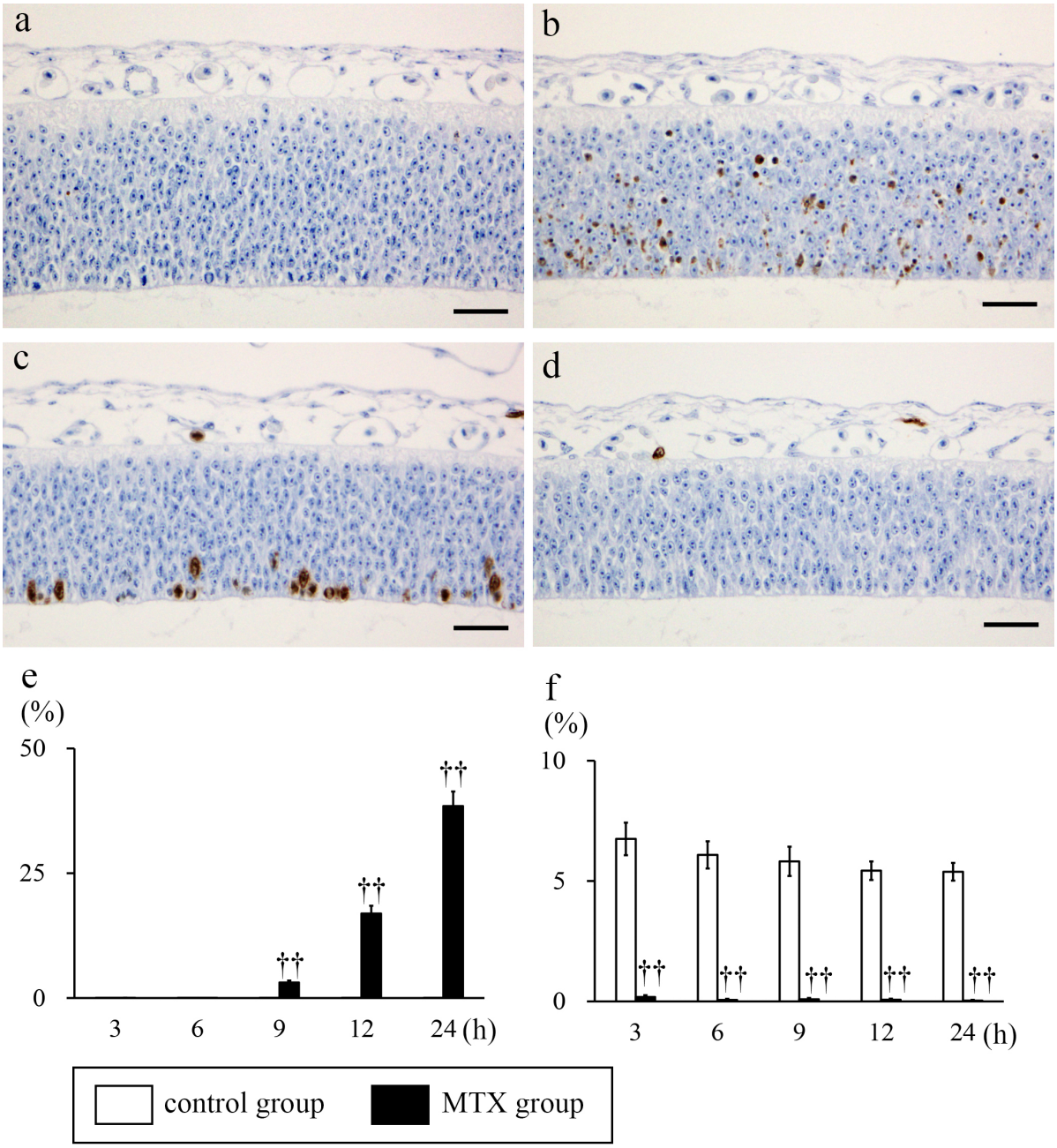

Fig. 3. Immunohistochemical analysis of cleaved caspase-3 and phospho-histone H3 expression in the ventricular zone of the optic tectum of Japanese quail embryos exposed to MTX.
(a, b) Immunohistochemical expression of cleaved caspase-3 in the ventricular zone of the optic tectum of Japanese quail embryos exposed to MTX. (a) Control embryo at 12 h after saline treatment. (b) MTX-treated embryo at 12 h after MTX treatment. Bar=30 μm.
(c, d) Immunohistochemical expression of phospho-histone H3 in the ventricular zone of the optic tectum of Japanese quail embryos exposed to MTX. (c) Control embryo at 3 h after saline treatment. (d) MTX-treated embryo at 3 h after MTX treatment. Bar=30 μm.
(e, f) Time course changes in cleaved caspase 3-positive rate (e), and phospho-histone H3-positive rate (f) in the ventricular zone of the optic tectum of Japanese quail embryos. Values are expressed as means ± SE. ††: Significantly different from the control group at $p<0.01$ (Welch's t-test).

exposure to 100 ng/g egg MTX on ED 4 induced apoptosis of neuroepithelial cells and inhibited proliferation of these cells in the ventricular zone of the optic tectum in Japanese quail embryos. These results indicated that neuroepithelial cells in the ventricular zone of the optic tectum at an early embryonic stage in Japanese quail embryos are sensitive to folic acid antimetabolites, such as MTX, and have a strong requirement for folic acid. A previous study demonstrated that neuroepithelial cells in the ventricular zone of the quail optic tectum at an early embryonic stage showed high cell proliferative activity[13], and the high cell proliferative activity of neuroepithelial cells in this period is considered to be involved in their high sensitivity to MTX.

The present study revealed that this in ovo exposed-Japanese quail embryo model reacted sensitively and rapidly to MTX. Considering that MTX inhibits DNA synthesis and it is classified as a DNA damaging agents[1–5], the results of the present study suggest the possibility that this model is useful as a simple and rapid screening evaluation for developmental neurotoxicity of DNA damaging compounds. To our knowledge, this is the first research paper suggesting the usefulness of an in ovo exposure assay using early-stage quail embryos as a developmental neurotoxicity assessment method of DNA damaging agents.

In the ventricular zone of the telencephalon in fetuses of rats and mice, the nuclei of proliferating neuroepithelial cells undergo a characteristic migration (interkinetic nuclear migration or elevator movement) in the ventricular zone, and the position of nuclei of neuroepithelial cells in the ventricular zone reflects their cell cycle phase[17, 18]. Previous studies demonstrated that in the ventricular zone of the telencephalon in fetuses of rats and mice exposed to chemicals, the difference in the distribution pattern of apoptotic neuroepithelial cells seems to reflect the differences in pharmacokinetics, mechanism of apoptosis and cell cycle arrest among the chemicals[18]. In the present study, apoptotic neuroepithelial cells were diffusely distributed throughout all layers of the ventricular zone in the optic tectum. However, the significance of the distribution pattern of apoptotic neuroepithelial cells in the ventricular zone of the optic tectum in avian embryos has been unclear, as research focusing on the histopathologic effects of chemicals on the optic tectum of avian embryos is lacking. It is necessary to accumulate knowledge about the distribution pattern of apoptotic neuroepithelial cells in the ventricular zone of the optic tectum in avian embryos and clarify its significance.

A previous study showed that the concentration of MTX in the surface waters was 0.044–5.0 ng/L[8], it is considered that its concentration is too low to induce directly histopathological changes, such as apoptosis of neuroepithelial cells and inhibition of their proliferation in the ventricular zone of the optic tectum in Japanese quail embryos. However, since many pharmaceuticals are designed to have high specificity and high affinity interactions with their biochemical target in the organism[19–21], there are concerns that MTX in the environment may have some adverse effects, such as genetic damages, on vertebrates populating there despite at low doses. Therefore, after all, the assessment of the environmental impact of MTX is important.

In conclusion, in ovo exposure to 100 ng/g egg MTX on ED 4 induced apoptosis of neuroepithelial cells and inhibited their proliferation in the ventricular zone of the optic tectum in Japanese quail embryos. While the detailed mechanism of MTX-induced neuroepithelial cell damage in the optic tectum of Japanese quail embryos remains unclear, the present results provide fundamental information about the risk assessment of MTX released into the environment.

Disclosure of Potential Conflicts of Interest: The authors declare that there is no conflict of interest.

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