

Time-course changes in neural cell apoptosis in the rat fetal brain from dams treated with 6-mercaptopurine (6-MP)

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Summary. 6-Mercaptopurine (6-MP), one of the major drugs for the therapy of acute lymphoblastic leukemia and autoimmune diseases, is incorporated as thioguanine in nucleic acid and it induces cytotoxicity and fetotoxicity. In the present study, pregnant rats were treated with 50 mg/kg of 6-MP on 13 embryonic days (E), and fetuses were collected from 12 to 96 h after the treatment to examine the mechanism and time-course changes in neural cell death in the developing brain. The weights of fetal telencephalon and the thickness of the dorsal telencephalic wall of the fetuses were significantly reduced at 96 h. The number of pyknotic neural cells in the fetal telencephalon began to increase at 24 h, peaked at 36 h, and then gradually decreased toward 72 h. The nuclei of most of these pyknotic cells were stained positively by TUNEL method, which detects DNA fragmentation. Moreover, pyknotic cells were immunohistochemically positive for cleaved caspase-3, one of the key executioners of apoptosis, and the increased expression of the protein from 30 to 48 h was confirmed by using Western blot analysis. Also, electron microscopical features of the pyknotic cells showed ultrastructural characteristics of apoptosis. On the other hand, the number of mitotic and BrdU-positive neural cells in the telencephalon decreased from 30 to 72 h. These results suggest that 6-MP induced apoptotic cell death in neural cells in the rat fetal brain is probably due to cytotoxic action of 6-MP.

Key words: Fetotoxicity, Apoptosis, Neural cell, 6-mercaptopurine, Fetal brain

Introduction

6-Mercaptopurine (6-MP) is used for the treatment of acute lymphoblastic leukemia and as an immunosuppressive drug (Estlin, 2001; Nielsen et al., 2001). 6-MP is first metabolized to an active metabolite after incorporation into cells. It inhibits several enzymes in the de novo purine synthetic pathway (Atkinson et al., 1964; McCollister et al., 1964; Shi et al., 1998). This active metabolite is further converted to thioguanine, which is incorporated into nucleic acid, and it induces cytotoxicity and fetotoxicity (Arakawa et al., 1967; Tidd and Paterson, 1974; Adhami and Noack, 1975).

Recently, more women have received this drug during pregnancy. It has been reported that human data include prematurity, low birth weight and malformations that occur especially when the drug is administered during pregnancy (Norgard et al., 2003). Also, investigations of rat fetuses obtained from dams treated with 6-MP have demonstrated central nervous system fetotoxicity (Arakawa et al., 1967; Adhami and Noack, 1975). Arakawa et al. (1967) reported that 6-MP induced microcephaly in the fetuses from dams which were intraperitoneally injected with 45 mg/kg on 8 embryonic days (E). However, there are no reports focusing on mechanism and time-course changes in neural cell death in the fetal rat central nervous system following 6-MP administration to their dams.

Therefore, the present study was carried out to clarify these points in the fetal brain obtained from pregnant rats treated with 6-MP on 13 E.

Material and methods

Animals and experimental design

About thirteen-week-old Pregnant rats of Crl:CD(SD) strain were purchased from Charles River Laboratories, Japan (Kanagawa, Japan). The animals were individually housed in wire-mesh cages in an airconditioned room (temperature, $23\pm 3^{\circ}\text{C}$; humidity, $50\pm 20\%$; ventilation, 10 times/hour; lighting, 12h light-12h dark cycle), and were given pelleted diet (CR-LPF, Oriental Yeast, Tokyo, Japan) and water *ad libitum*. 6-MP (LKT Laboratories, St. Paul, MN, USA) was dissolved in 2.0% methylcellulose solution in distilled water, and 5-bromo-2'-deoxyuridine (BrdU) (Sigma, St. Louis, MO, USA) was dissolved in saline.

In the first experiment, twenty-one pregnant rats were injected i.p. with 50 mg/kg of 6-MP on 13 E, and three dams were sacrificed by exsanguinations from abdominal aorta under ether anesthesia at 12, 24, 30, 36, 48, 72, and 96 h after the treatment, respectively. Fetuses were collected from dams by Caesarian section. As controls, twenty-one pregnant rats were injected i.p. with 2.0% methylcellulose solution in distilled water on 13 E, and three dams each were sacrificed in the same way. All rats were injected i.p. with 20 mg/kg of BrdU exactly 1 hour before sacrifice. In the second experiment, the same protocol as mentioned in the first experiment was used and the telencephalons of five fetuses from each dam were used for Western blot analysis.

The protocol of the present experiments was conducted according to the Guidelines for Animal Experimentation, the Japanese Association for Laboratory Animal Science, 1987.

Histopathology

The fetuses were fixed in 10% neutral-buffered formalin. Two- μm paraffin sections were stained with hematoxylin and eosin (HE) for histopathological examination.

In situ detection of fragmented DNA

DNA fragmentation was examined on the paraffin sections by the modified terminal deoxynucleotidyltransferase-mediated dUTP end labeling (TUNEL) method using a commercial apoptosis detection kit (ApopTag In situ Apoptosis Detection Kit; Chemicon, Temecula, CA, USA). In brief, the procedure was as follows: multiple fragmented DNA 3'-OH ends on the sections were labeled with digoxigenin-dUTP in the presence of terminal deoxynucleotidyl transferase (TdT). Peroxidase-conjugated anti-digoxigenin antibody was then reacted with the sections. Apoptotic nuclei were visualized by peroxidase-diaminobenzidine (DAB) reaction. The sections were then counterstained with methyl green.

Immunohistochemistry

For the detection of cleaved caspase-3, paraffin sections were deparaffinized and immersed in 10 mM citrate buffer, pH 6.0, and heated at 121°C for 15 min by autoclaving. After washing in Tris-buffered saline (TBS), the sections were then placed in 0.3% H_2O_2 -containing methanol for 30 min to inactivate endogenous peroxidase. The sections were incubated in skimmed milk at room temperature for 2 h to reduce nonspecific staining, and reacted with rabbit anti-cleaved caspase-3 antibody (Cell Signaling Technology, Beverly, MA, USA) at 4°C overnight. They were reacted with biotin labeled anti-rabbit IgG (KPL, Gaithersburg, MD, USA) at room temperature for 40 min, and then with peroxidase-labeled streptavidin (DAKO, Carpinteria, CA, USA) at room temperature for 40 min. For the detection of BrdU, sections were treated with 0.1% trypsin and 0.1% calcium chloride in Tris buffer at 37°C for 30 min, and then with 1N HCl at room temperature for 45 min. After washing in TBS, the sections were placed in 0.3% H_2O_2 -containing methanol for 30 min, and incubated in skimmed milk at room temperature for 2 h. They were reacted with mouse anti-BrdU antibody (DAKO) at 4°C overnight, with biotin labeled anti-mouse IgG (KPL) at room temperature for 40 min, and then with peroxidase-labeled streptavidin (DAKO) at room temperature for 40 min. The positive signals were visualized by DAB reaction. The sections were counterstained with methyl green.

Protein extraction and Western Blot

Five fetal telencephalons were collected from each dam. The telencephalons were homogenized in 10 mM Tris-HCl buffer (pH 7.8) containing 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10 mM NaF, 2 mM Na_3VO_4 , and Proteinase Inhibitor Cocktail (Roche Applied Science, Penzberg, Germany), and used as a whole-cell lysate. Protein concentrations were determined with a Lowry protein assay kit (Bio-Rad, Hercules, CA, USA).

Equal amounts of protein were loaded onto a SDS-PAGE gel, electrophoresed, and transferred to a PVDF membrane (Bio-Rad). The blotted membranes were probed with antibodies to cleaved caspase-3 (diluted 1000-fold, Cell Signaling Technology), β -actin (diluted 1000-fold, Cell Signaling Technology). After incubation with the appropriate secondary antibodies conjugated to horseradish peroxidase (Amersham, Buckinghamshire, UK), positive immunoreactivity was visualized with ECL Plus Western Blotting Detection Reagent (Amersham), and detected with ChemDoc XRS-J (Bio-Rad). After the detection, to ensure that each lane contained a similar amount of protein, the membranes were stripped and then reprobed with primary antibodies against β -actin.

For quantification of the Western blot analyses, the immunoreactive bands were quantified by densitometry

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and the expression levels calculated relative to the levels of β -actin in each protein sample; the fold changes relative to the values in the control groups were represented as the means \pm SD for three independent experiments.

Electron microscopy

Small pieces of the fetal brain were fixed in 1.5% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed in 1% osmium tetroxide, and then embedded in Epon 812 resin (TAAB, Reading, UK). Semithin sections were stained with toluidine blue for light microscopic survey. Ultra-thin sections of selected areas were then double-stained with uranyl acetate and lead citrate, and observed under a JEM-100CX II transmission electron microscope (Nippon Denshi, Tokyo, Japan).

Morphometry

Pyknotic or mitotic neural cells in the ventricular zone (VZ) of the telencephalon were counted in three

randomly chosen fetuses/dam, and from 3 dams on HE-stained sections under a light microscope. Three hundred cells were counted in each fetus. TUNEL-, cleaved caspase-3- or BrdU-positive cells were counted in the same way on immunostained sections. Moreover, the thickness of the dorsal telencephalic wall was measured on HE-stained sections in the same way.

Statistical analysis

Fetal weight, thickness of the telencephalic wall, and percentages of pyknotic cells, mitotic cells, TUNEL-positive cells, cleaved caspase-3-positive cells and BrdU-positive cells were expressed as the mean \pm standard deviation (SD), respectively, at each point of examination. Statistical analysis was carried out by Student's *t*-test.

Results

Pyknotic cells were observed in the diencephalon (Fig. 1b), mesencephalon (Fig. 1c), metencephalon (Fig. 1d) and spinal cord (Fig. 1e) of the 6-MP treated fetuses,

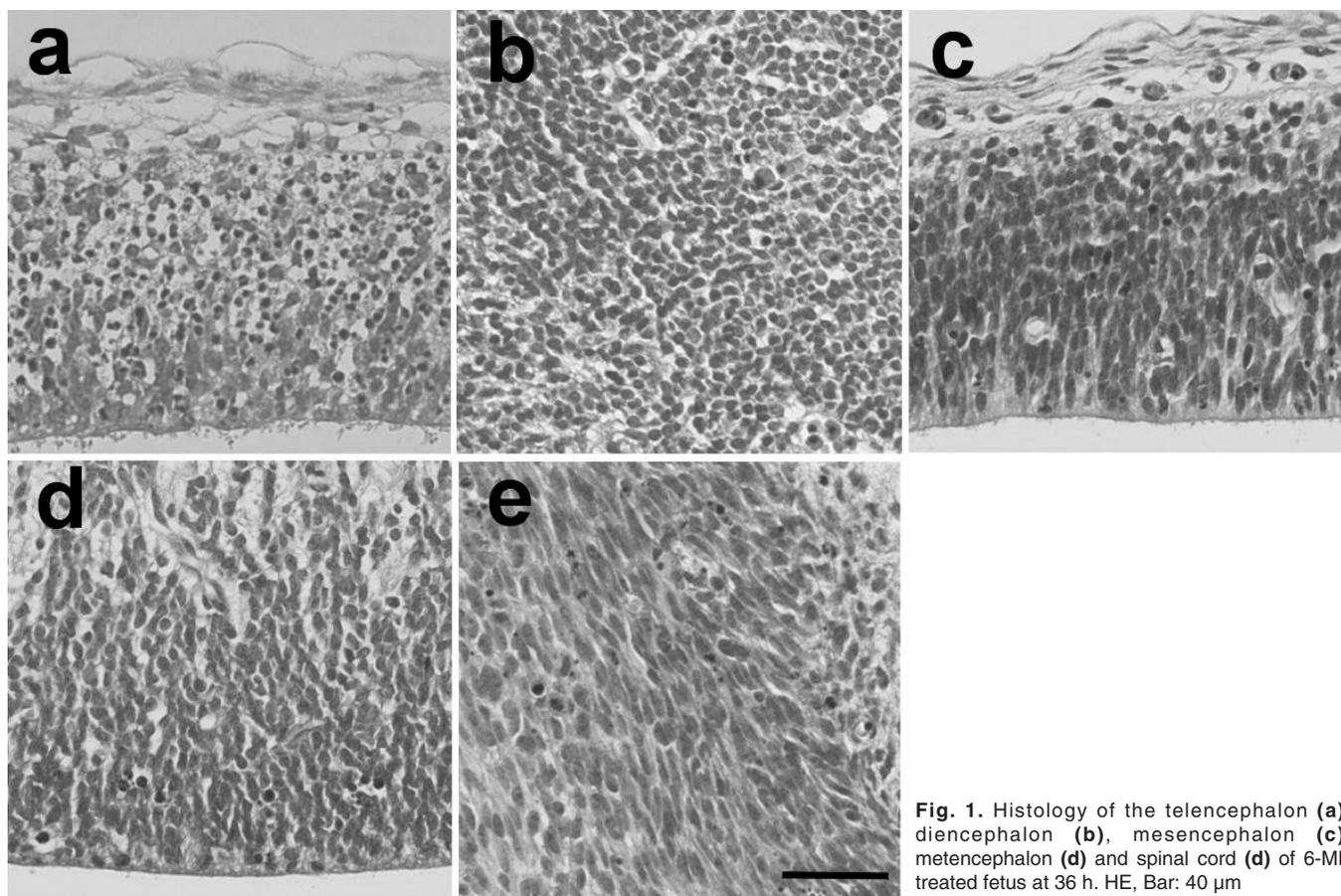


Fig. 1. Histology of the telencephalon (a), diencephalon (b), mesencephalon (c), metencephalon (d) and spinal cord (e) of 6-MP treated fetus at 36 h. HE, Bar: 40 μ m

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while the lesions were much less prominent than those in the telencephalon (Fig. 1a). Thus, here we investigated the effect on fetal telencephalon obtained from pregnant rats treated with 6-MP on 13 E.

Compared with the control group, the weight of the

telencephalon and the thickness of the dorsal telencephalic wall of the 6-MP treated fetuses were significantly reduced at 96 h (Fig. 2).

The number of Pyknotic neural cells in the fetal telencephalon began to increase at 24 h (Fig. 3Ab),

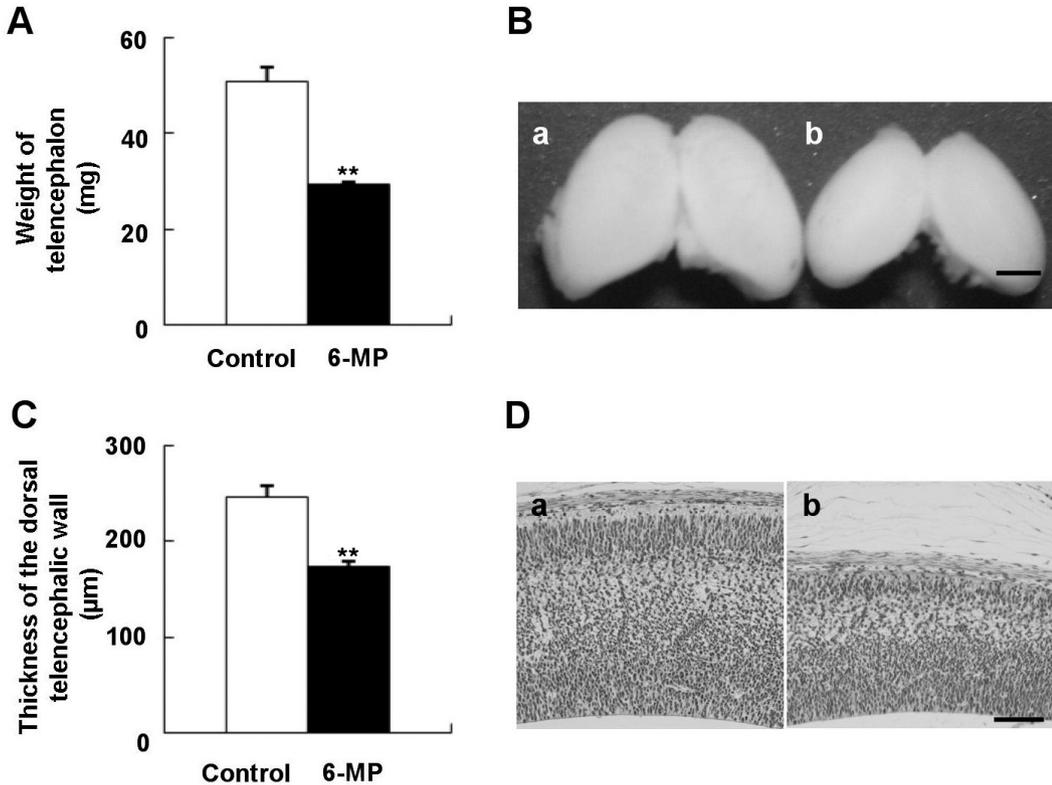


Fig. 2. **A.** Weight of telencephalon of the fetal brain at 96 h. **B.** The telencephalon of a control fetus (**a**) and that of a 6-MP treated fetus (**b**) at 96 h. **C.** Thickness of the telencephalic wall of the fetal brain at 96 h. **D.** The telencephalic wall of a control fetus (**a**) and that of a 6-MP treated fetus (**b**) at 96 h (HE). Each value represents the mean \pm SD of 3 dams. **P<0.01. Bar: B, 1 mm; D, 50 μ m

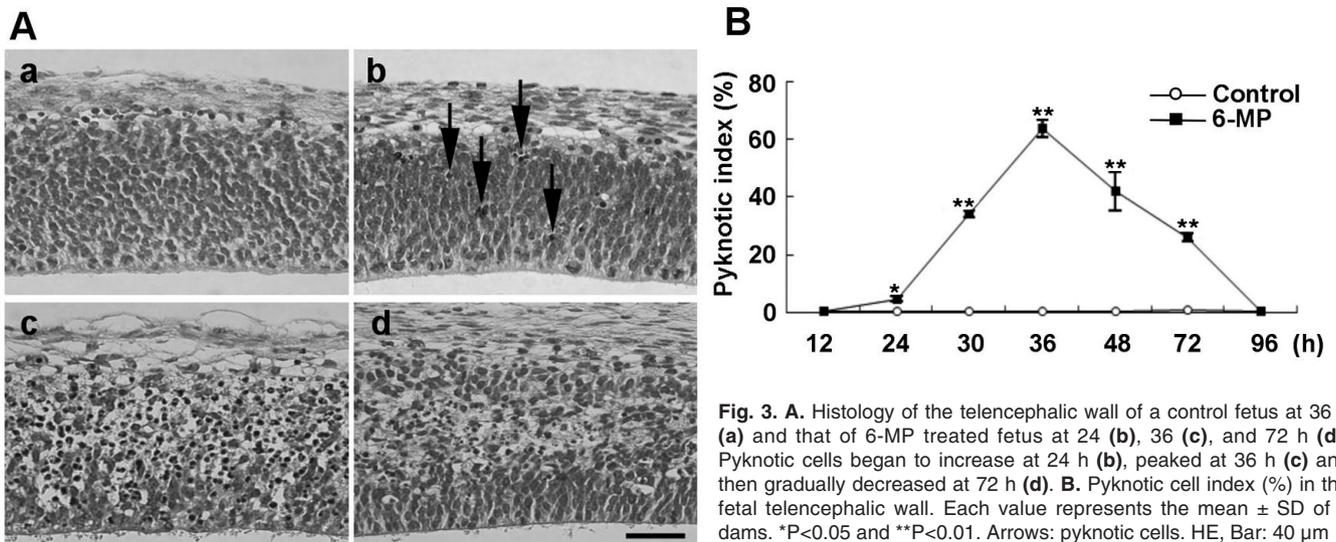


Fig. 3. **A.** Histology of the telencephalic wall of a control fetus at 36 h (**a**) and that of 6-MP treated fetus at 24 h (**b**), 36 h (**c**), and 72 h (**d**). Pyknotic cells began to increase at 24 h (**b**), peaked at 36 h (**c**) and then gradually decreased at 72 h (**d**). **B.** Pyknotic cell index (%) in the fetal telencephalic wall. Each value represents the mean \pm SD of 3 dams. *P<0.05 and **P<0.01. Arrows: pyknotic cells. HE, Bar: 40 μ m

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peaked at 36 h (Fig. 3Ac), gradually decreased toward 72 h (Fig. 3Ad), and returned to the control level at 96 h (Fig. 3B). Pyknotic cells were found mainly diffusely distributed throughout VZ (Fig. 3A).

Most of the pyknotic neural cells in the telencephalon of 6-MP treated fetuses were positively stained with TUNEL, and the increase in the number of such TUNEL-positive cells (Fig. 4A,B) coincided with that of pyknotic cells. The number of cleaved caspase-3-positive cells in the fetal telencephalon increased from 24 h (Fig. 5Ab), peaked at 36 h (Fig. 5Ac), and then returned to the control level at 96 h (Fig. 5B). Also,

western blot analysis confirmed that the expression of cleaved caspase-3 protein increased from 30 to 48 h (Fig. 6A, B).

Electron-microscopically, pyknotic cells were characterized by the shrinkage of the cell body, condensation and/or margination of nuclear chromatin (Fig. 7a). Some pyknotic cells were fragmented into small pieces, which were frequently ingested by adjacent cells (Fig. 7b).

Mitotic cell index in the ventricular surface began to decrease at 30 h, reached the minimal level at 36 h (Fig. 8Ab), and then recovered to the control level at 96 h

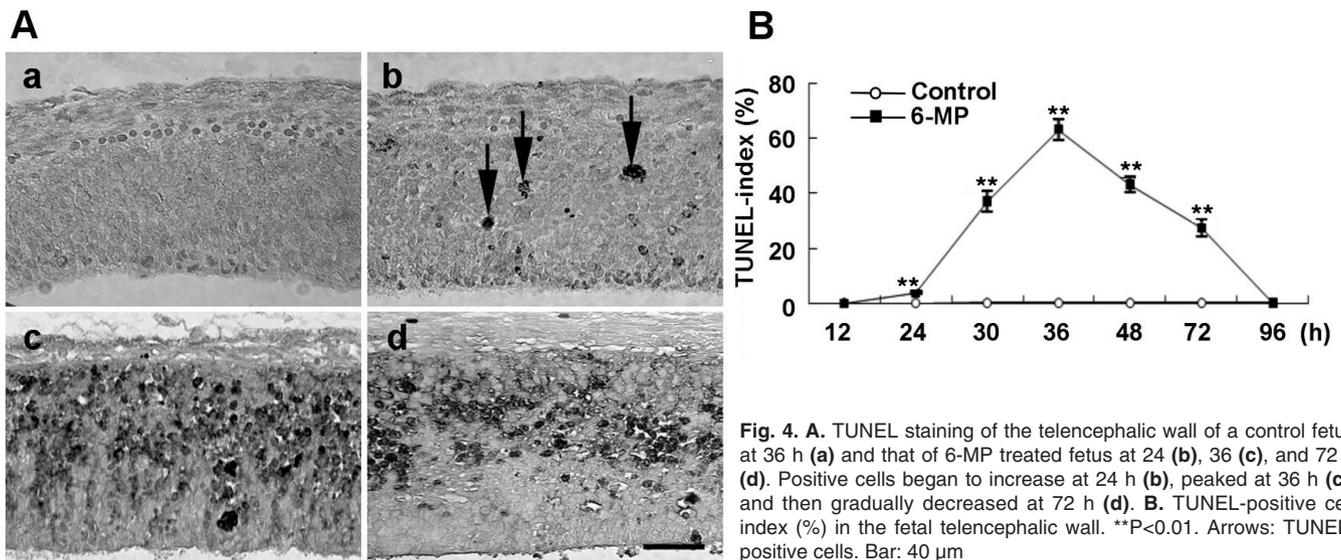


Fig. 4. A. TUNEL staining of the telencephalic wall of a control fetus at 36 h (a) and that of 6-MP treated fetus at 24 (b), 36 (c), and 72 h (d). Positive cells began to increase at 24 h (b), peaked at 36 h (c), and then gradually decreased at 72 h (d). **B.** TUNEL-positive cell index (%) in the fetal telencephalic wall. ** $P < 0.01$. Arrows: TUNEL-positive cells. Bar: 40 μ m

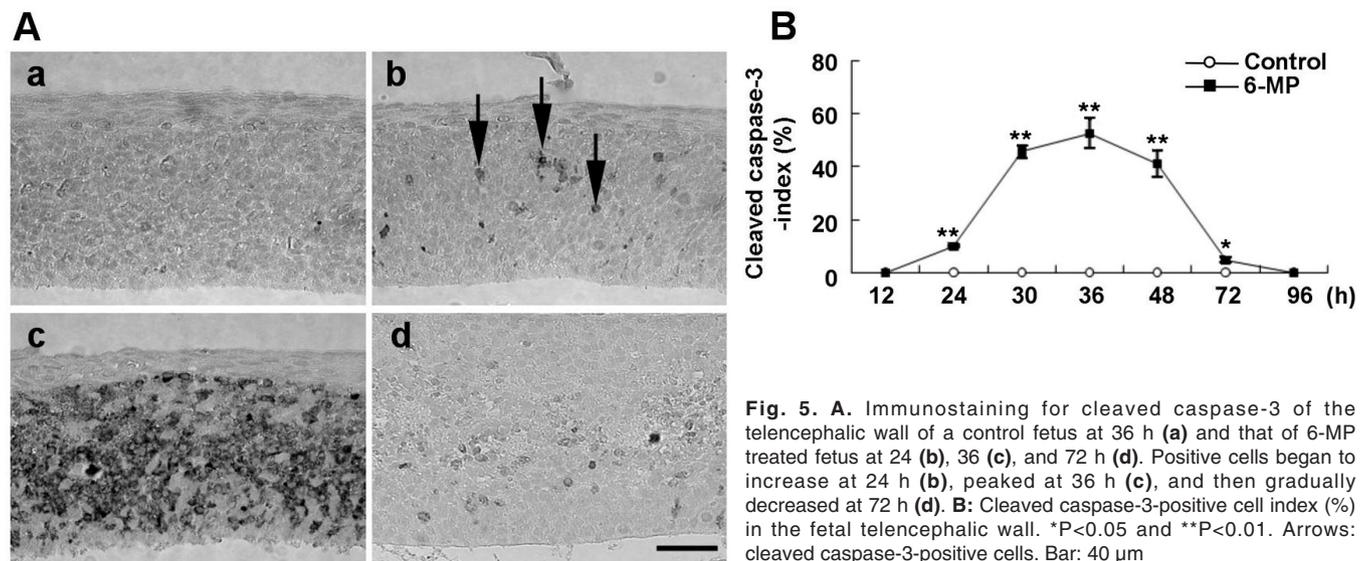


Fig. 5. A. Immunostaining for cleaved caspase-3 of the telencephalic wall of a control fetus at 36 h (a) and that of 6-MP treated fetus at 24 (b), 36 (c), and 72 h (d). Positive cells began to increase at 24 h (b), peaked at 36 h (c), and then gradually decreased at 72 h (d). **B.** Cleaved caspase-3-positive cell index (%) in the fetal telencephalic wall. * $P < 0.05$ and ** $P < 0.01$. Arrows: cleaved caspase-3-positive cells. Bar: 40 μ m

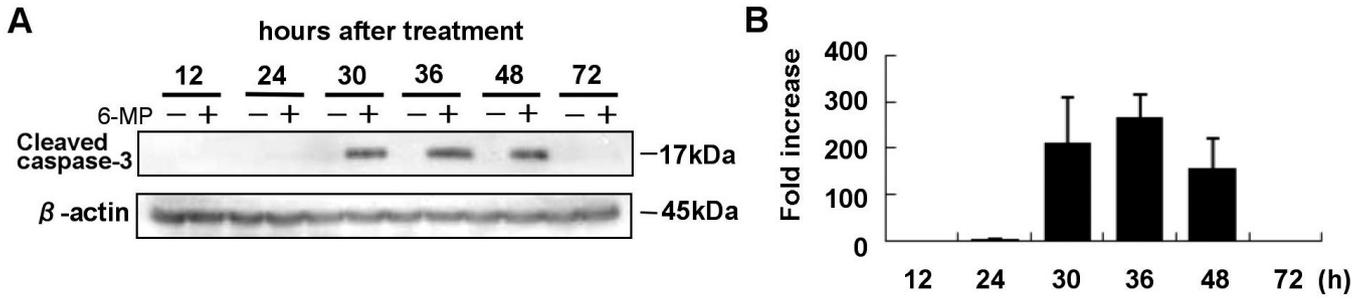


Fig. 6. A. Western blot analysis of cleaved caspase-3. **B.** Quantitative change in the expression of cleaved caspase-3 protein in the 6-MP treated fetal telencephalon. β -actin was used as a loading control. Each value represents the mean \pm SD of 3 dams.

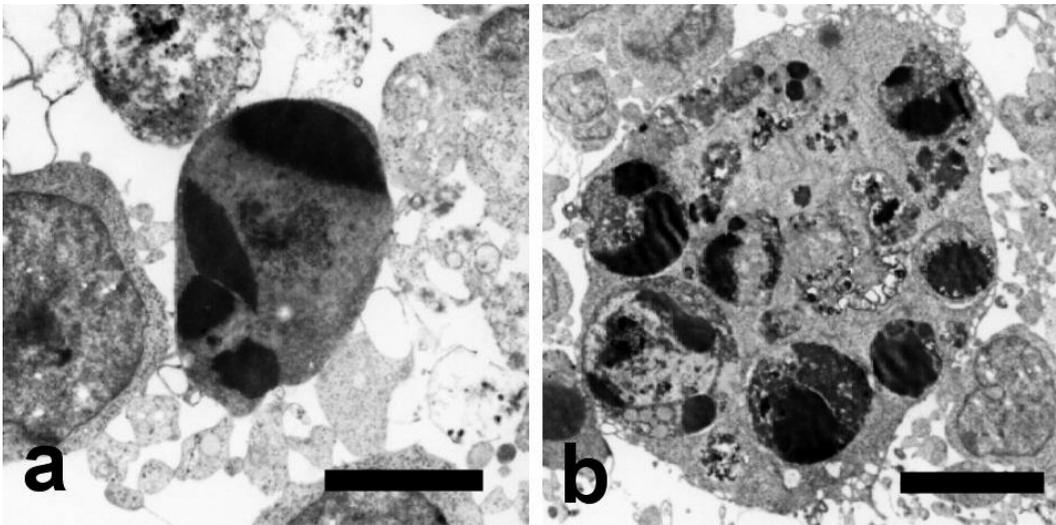


Fig. 7. Electron microscopy of neural cells in the telencephalic wall of a 6-MP treated fetus at 36 h. Condensation and margination of nuclear chromatin (a) and apoptotic bodies are ingested by an adjacent cell (b). Bar: a, 3 μ m; b, 5 μ m

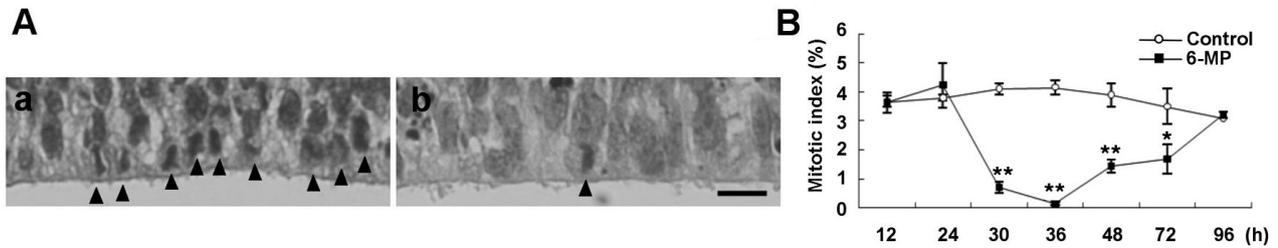


Fig. 8. A. Mitotic cells in the ventricular surface of control fetus (a) and that of 6-MP treated fetus (b) at 36 h. **B.** Mitotic cell index (%) in the fetal ventricular surface. * $P < 0.05$ and ** $P < 0.01$. Arrowheads: mitotic cells. HE, Bar: 20 μ m

(Fig. 8B). BrdU-positive cell index in the telencephalon also showed similar time-course changes of mitotic cell index (Fig. 9 A,B).

Discussion

The present study was carried out to clarify the

mechanism and time-course changes in neural cell death in the fetal rat central nervous system following 6-MP administration to their dams on 13 E.

The nuclei of the majority of the pyknotic cells were positive for TUNEL and cleaved caspase-3. In western blot analysis, the increment of cleaved caspase-3 protein was in close agreement with its immunohistochemistry.

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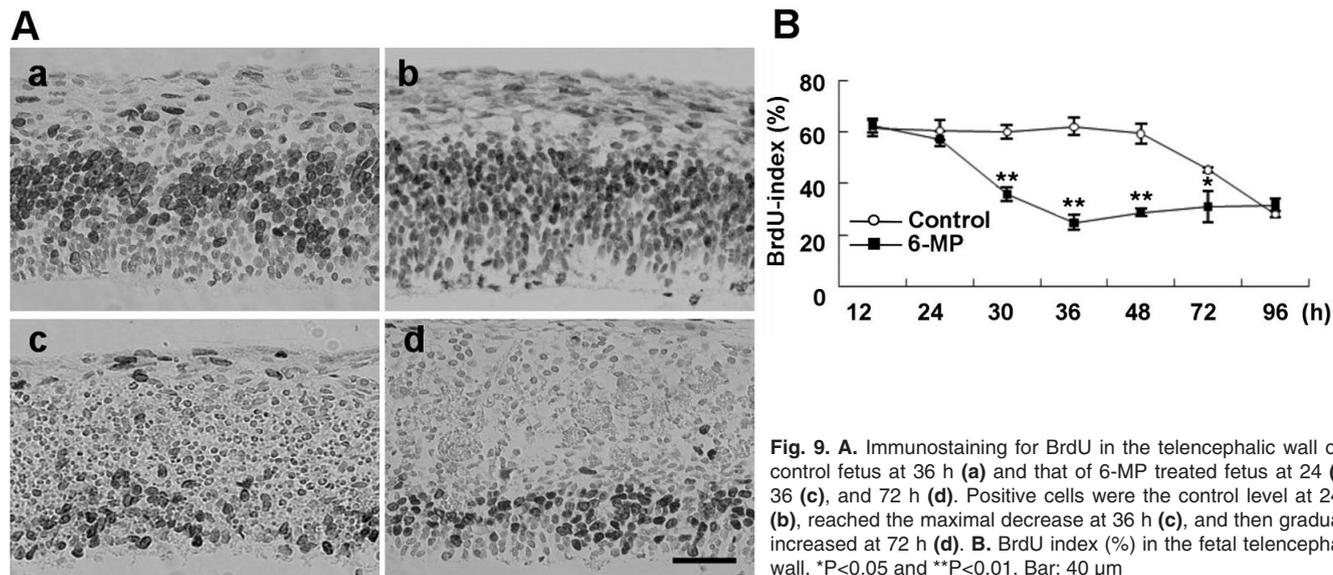


Fig. 9. A. Immunostaining for BrdU in the telencephalic wall of a control fetus at 36 h (a) and that of 6-MP treated fetus at 24 h (b), 36 h (c), and 72 h (d). Positive cells were the control level at 24 h (b), reached the maximal decrease at 36 h (c), and then gradually increased at 72 h (d). **B.** BrdU index (%) in the fetal telencephalic wall. * $P < 0.05$ and ** $P < 0.01$. Bar: 40 μ m

Activation of caspase-3 is known to be involved in neural cell apoptosis during the normal fetal brain development, as well as in that induced by DNA-damaging agents (Kuida et al., 1996; Keramaris et al., 2000). Moreover, in the present study, electron-microscopic features of these pyknotic cells were identical to the morphological characteristics of apoptotic cells (Ihara et al., 1998). These results indicated that pyknotic cells observed in the developing brain of the 6-MP treated rat fetuses are suffering from apoptosis.

In contrast to the increment of apoptotic neural cell, a decrease in the numbers of mitotic and BrdU-positive neural cells were observed in the fetal rat brain treated with 6-MP. It has been reported that 6-MP depressed proliferating activity (Bokkerink et al., 1993; Worting and Roti, 1980). We may suggest that 6-MP depressed proliferating activity of neural cells in the fetal telencephalon. In this study, however, we could not exactly confirm the presence or absence of indicators that 6-MP depressed proliferating activity of neural cells in the fetal telencephalon. Further studies are needed to discuss this point in more detail.

Increased apoptosis in fetal neural cells was also reported in rats and mice following prenatal treatment with DNA damaging agents, such as 5-azacytidine (Lu et al., 1998; Ueno et al., 2002), ethylnitrosourea (Katayama et al., 2000), etoposide (Nam et al., 2006), hydroxyurea (Woo et al., 2003) and 1- β -D-arabinofuranosylcytosine (Yamauchi et al., 2004). Although these agents induced neural cell apoptosis from 3 to 24 h, 6-MP induced apoptosis from 24 to 72 h. Thus, these studies indicate that cytotoxic action of 6-MP was prominently slower in the time-course of apoptotic neural cell than other DNA damaging agents, which were used in similar experimental systems.

However, the precise mechanism of cytotoxic action of 6-MP still remains unclear. Further studies are needed.

In conclusion, neural cell death in the fetal rat brain from dams treated with 6-MP was apoptotic neural cell death, and the time-course of apoptotic neural cell was remarkably slower than other DNA damaging drugs. The present results offer a key for studies on the mechanisms of fetotoxicity induced by 6-MP. Further studies on gene levels are necessary to elucidate the molecular pathway of 6-MP-induced fetotoxicity.

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