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# Apoptotic cell death and cell proliferative activity in the rat fetal central nervous system from dams administered with ethylnitrosourea (ENU)

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**Summary.** Ethylnitrosourea (ENU), a well known DNA alkylating agent, induces anomalies in the central nervous system (CNS), craniofacial tissues and male reproductive organs, and the enhancement of apoptosis is found in these tissues immediately after the administration of ENU (Katayama et al., 2000a). In this study, pregnant rats were treated with 60mg/kg of ENU at day 13 of gestation, and kinetics of apoptotic cells, mitotic cells and bromodeoxyuridine (BrdU)-positive cells in the fetal CNS were examined from 3 to 48 hours after the treatment (HAT). From 3 HAT, a significant increase in the number of apoptotic cells and a significant decrease in the number of mitotic cells were detected in the fetal CNS, and BrdU-positive cells significantly decreased in accordance with the increase in the number of apoptotic cells. The present results strongly suggest that both excess cell death by apoptosis and cell growth arrest indicated by decreased number of mitotic cells and BrdU-positive cells may have a close relation to the later occurrence of microencephaly following ENU-administration, and that ENU affects mainly S-phase cells and causes apoptosis.

**Key words:** Apoptosis, Cell growth arrest, Ethylnitrosourea, Fetus, Rat

## Introduction

Ethylnitrosourea (ENU), a well known DNA alkylating agent, induces brain tumors in offsprings when administered to pregnant rats (Druckrey et al., 1967; Bosch, 1977a). ENU is also known as a teratogen, and it induces anomalies in the central nervous system (CNS) (Pfaffenroth et al., 1974; Hallas and Das, 1978, 1979; Oyanagi et al., 1988), craniofacial tissues (Ohnishi, 1989), limbs (Druckrey et al., 1967) and male reproductive organs (Nagao et al., 1996). Recently, we revealed for the first time that ENU-administration to pregnant rats at day 13 of gestation (GD13) caused a significant increase in the number of apoptotic cells in the fetal CNS, craniofacial mesenchymal tissues, limb bud and tail bud (Katayama et al., 2000a) and reported that ENU-administration to dams induced anomalies in these tissues in offsprings (Katayama et al., 2000b). In the fetal rat CNS, it is also reported that ENU causes cell growth arrest (Bosch, 1977b; Fujiwara, 1980; Oyanagi et al., 1998).

In this study, we examined the changes in the apoptotic cell death and in the cell proliferative activity in the rat fetal CNS after ENU-administration to pregnant rats at GD13, which is reported to be the most sensitive day of the rat fetal CNS to ENU-administration (Pfaffenroth et al., 1974; Hallas and Das, 1978), in order to find a clue to clarify the more detailed mechanisms of ENU-induced fetotoxicity and teratogenicity in the CNS.

All procedures including animals were performed in accordance with the protocol approved by the Animal Care and Use Committee of Graduate School of Agricultural and Life Sciences, The University of Tokyo.

## Materials and methods

### Animals

Forty pregnant Jcl:F344 rats (plug day : day 0 of gestation) were obtained from Saitama Experimental Animal Co, Saitawa, Japan. They were kept udner controlled conditions (temperature, 23±2 °C; relative humidity, 55±5%) using an isolator caging systems and were fed commercial pellets (MF, Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*.

### Chemicals

N-Nitroso-N-Ethylurea (ENU) (Sigma, St. Louis,

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MO) was dissolved in 2mM sodium citrate buffer (pH4.5) immediately before the treatment, and the concentration was adjusted to 10mg/ml. 5-bromo-2'-deoxyuridine (BrdU) (Sigma, St. Louis, MO) was dissolved in saline immediately before the treatment, and the concentration was adjusted to 5mg/ml.

### *Treatments*

Twenty-five pregnant rats were injected with 60mg/kg of ENU intraperitoneally (i.p.) at day 13 of gestation (GD13), and five dams were sacrificed by heart puncture under ether anesthesia at 3, 6, 12, 24 and 48 hours after the treatment (HAT), respectively. The remaining fifteen pregnant rats were injected i.p. with citrate buffer alone at GD13, and three dams were sacrificed in the same way at 3, 6, 12, 24 and 48 HAT, respectively. All rats were injected i.p. with 20mg/kg of BrdU exactly 1 hour before sacrifice.

### *Histopathology*

Fetuses were collected by Caesarian section and fixed in 10% neutral-buffered formalin. Paraffin sections (4 $\mu$m) were stained with hematoxylin and eosin (HE). Some of the paraffin sections were subjected to in situ detection of fragmented DNA as mentioned below.

### *In situ detection of fragmented DNA*

DNA fragmentation was examined on the paraffin sections by the modified TUNEL method first proposed by Gravieli et al. (1992), with commercial apoptosis detection kit (ApopTag In situ Apoptosis Detection Kit; Intergene, Purchase, NY). 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 methylgreen.

### *Electron microscopy*

Small pieces of fetal brains were fixed in 2% glutaraldehyde in 0.1M phosphate buffer (pH7.4) and postfixed in 1% osmium tetroxide in the same buffer, and embedded in Epok 812 (Oken, Tokyo, Japan). Semithin sections were stained with toluidine blue for light microscopic survey. Ultrathin sections of the selected areas were double-stained with uranyl acetate and lead citrate and observed under a JEOL 1200 EX electron microscope (Nippon Denshi, Tokyo, Japan).

### *Immunohistochemical staining for BrdU*

To evaluate the proliferative activity of neuroepithelial cells in the rat fetal central nervous system (CNS), immunohistochemical staining for BrdU was carried out on the paraffin sections by the LSAB method with streptavidine (Dako, Carpinteria, CA). Mouse anti-BrdU monoclonal antibody (Amersham, Bucks, UK) was used as the primary antibody. The sections were visualized by peroxidase-diaminobenzidine (DAB) reaction and then counterstained with methylgreen.

### *Morphometry*

Apoptotic and mitotic neuroepithelial cells in the dorsal telencephalic wall were counted in two randomly chosen fetuses from a dam on the HE-stained sections under light microscope. 500 cells were counted in each fetus. BrdU-positive cells were also counted in the same way on the immunohistochemically stained sections. Thickness of the dorsal telencephalic wall was measured in two randomly chosen fetuses from a dam at 48 HAT on the HE-stained sections under light microscope.

The apoptotic, mitotic, BrdU-labeling indices (%) and thickness of the telencephalic wall were expressed as the mean±standard deviation (SD) of 5 dams for the ENU-treated group and 3 dams for the control group, respectively, and statistical analysis was carried out by Student's t-test.

## Results

Apoptotic cell death characterized by pyknotic nuclei and/or detected by the TUNEL method was found in neuroepithelial cells of the fetal CNS from 3 HAT. The number of apoptotic cells peaked at 12 HAT, gradually decreased towards 24 HAT, and returned to the control level at 48 HAT. Apoptosis in the CNS was mainly observed in the neuroepithelial cells beneath the external limiting membrane of the ventricular layer. On the other hand, only a few apoptotic cells were seen in the CNS of control fetuses throughout the experimental period (Figs. 1-3).

The number of mitotic cells decreased from 3 HAT, and reached the lowest level at 12 HAT. Mitotic index again increased at 24 HAT, and reached the level above the control one at 48 HAT (Figs. 1, 4).

The number of BrdU-positive cells gradually decreased from 3 HAT and reached the minimum level at 6 HAT. At 48 HAT, BrdU-labeling index returned to the control level (Figs. 5, 6).

Kinetics of the neuroepithelial cells including apoptotic cells, BrdU-positive cells and mitotic cells in the telencephalic wall are summarized in Fig. 7.

Electron microscopically, the pyknotic cells were characterized by shrinkage of the cell body, condensation of nuclear chromatin and/or margination of condensed chromatin along the nuclear membrane (Fig. 8). Some nuclei were fragmented into small pieces, which were frequently ingested by adjacent macrophages.

At 48 HAT, thickness of the telencephalic wall of the

ENU-group was significantly smaller than that of the control group (Fig. 9).

## Discussion

In this study, apoptotic cell death and cell growth arrest were observed in the rat fetal CNS after ENU-administration to dams as reported in other papers (Bosch, 1977b; Fujiwara, 1980; Oyanagi et al., 1988, 1998; Katayama et al., 2000a), and thickness of the dorsal telencephalic wall of the ENU-group was significantly smaller than that of the control group. Thus both apoptotic cell death and cell growth arrest were thought to play a crucial role in the fetotoxicity and teratogenicity of ENU.

In the CNS of ENU-group, apoptosis was most

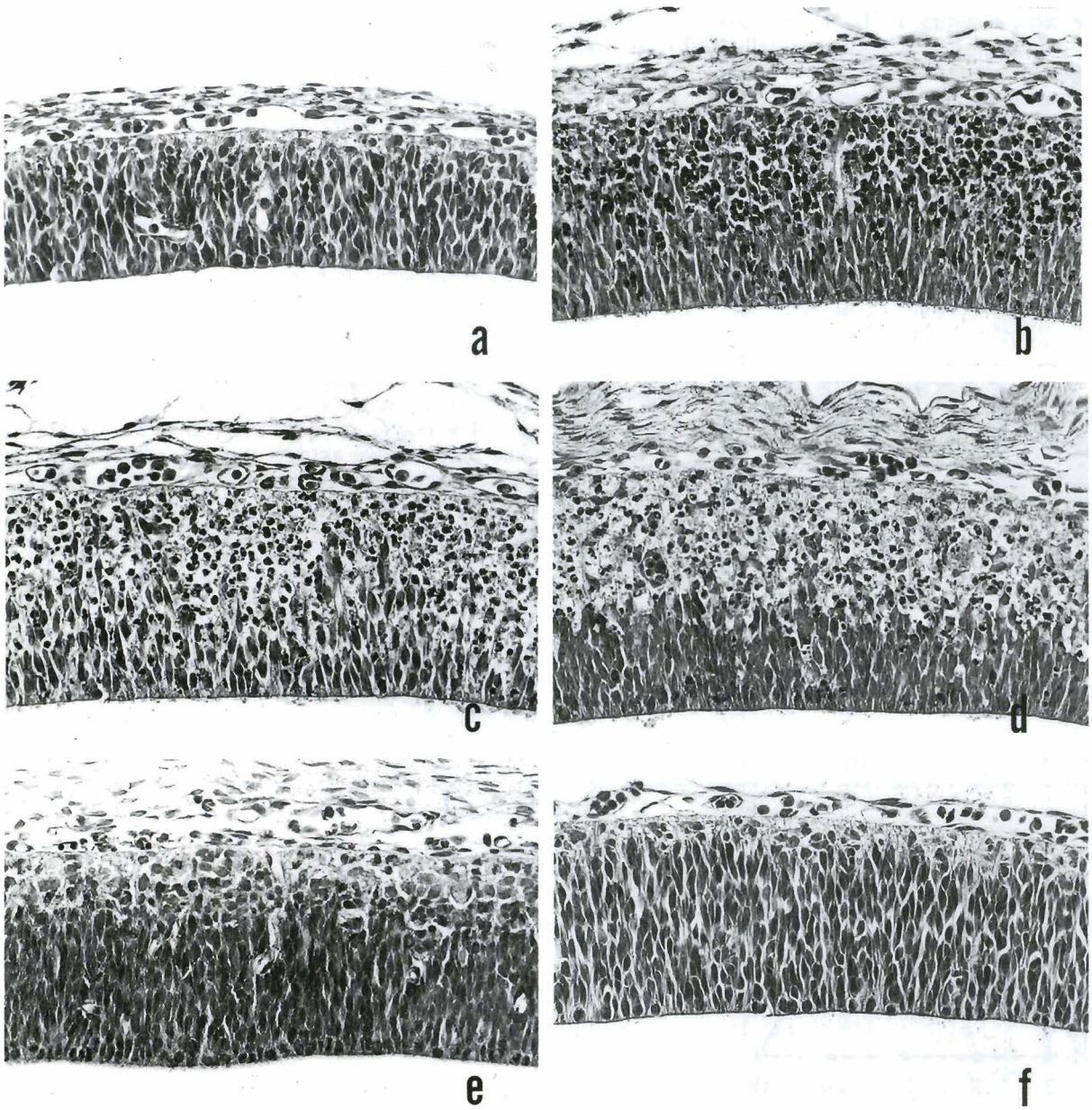


**Fig. 1**. Histological appearances of the dorsal telencephalic wall of the ENU-group at 3 **(a)**, 6 **(b)**, 12 **(c)**, 24 **(d)** and 48 HAT **(e)** and of the control group at 12HAT **(f)**. HE. Apoptotic cells were found from 3 HAT. The number of apoptotic cells peaked at 12 HAT, gradually decreased towards 24 HAT, and returned to the control level at 48 HAT. Apoptosis in the CNS was mainly observed in the neuroepithelial cells beneath the external limiting membrane of the ventricular layer. The number of mitotic cells decreased from 3 HAT, and reached the lowest level at 12 HAT. Mitotic index again increased at 24 HAT, and reached the level above the control one at 48 HAT. x 270

frequently seen in the neuroepithelial cells beneath the external limiting membrane of the ventricular layer, and the number of BrdU-positive cells decreased in accordance with the increase in the number of apoptotic cells at 6 HAT. It is said that the neuroepithelial cells beneath the external limiting membrane actively synthesize DNA (Langman et al., 1966), and that ENU damages cellular DNA synthesis by alkylation of the bases and is decomposed with a half-life of less than 10 minutes in the intact animal (Swann and Magee, 1971; Itze et al., 1979). Thus, ENU seems to affect mainly the S-phase cells, resulting in the induction of apoptosis. A similar enhancement of apoptotic cell death was also reported in the fetal CNS of rats following administration of 5-azacitidine, a DNA hypomethylating agent (Lu et al., 1998), and following γ-radiation (Borovitskaya et al., 1996). These findings suggest that the fetal CNS may be highly sensitive to such genotoxic stimuli and easily cause apoptosis. Apoptotic reaction to ENU-treatment was slower than that to γ-radiation and faster than that to 5-azacitidine-treatment. These differences in apoptotic reaction may reflect the differences in the toxic effects of these stimuli on neuroepithelial cells. Direct comparative studies on these different stimuli may be useful for a further understanding of the mechanisms of fetotoxicity and teratogenicity in the CNS.

The time of onset of recovery in the number of mitotic cells (at 24 HAT) was delayed about 12 hours more than that in the number of BrdU-positive cells (at 12 HAT). This time lag may be explicable by the fact that S-phase of the neuroepithelial cells around GD13 continues about 6 to 8 hours in the normal rat fetal CNS (Weachter and Jaensch, 1972)

At 48 HAT, BrdU-labeling index recovered to the control level, while the mitotic index was higher in the ENU-group than in the control group. Yamano et al. (1981) reported that shortening of the generation time was observed in the external granular cell layer of the cerebellum of neonatal mice in the recovery phase after destruction by administration of cytosine arabinoside, a radiomimetic substance. The shortening of the generation time seems to occur also in the neuroepithelial cells in the fetal CNS at 48 HAT. To clarify this point, further investigations of the effects of ENU-administration on the cell cycle of neuroepithelial cells in the fetal CNS are necessary.

The degree of apoptotic cell death was severer and the regenerative activity appeared later in the present study compared with the report of Oyanagi et al. (1998) in which ENU-treatment was done at GD15. This well corresponds to the reports that ENU-administration at GD13 cause severer microencephaly than that at GD15 (Pfaffenroth et al., 1974; Hallas and Das, 1978)

The number of chemicals with teratogenic potency is increasing year by year in the environment surrounding humans and animals, and therefore the clarification of mechanisms of fetotoxicity and teratogenicity of these

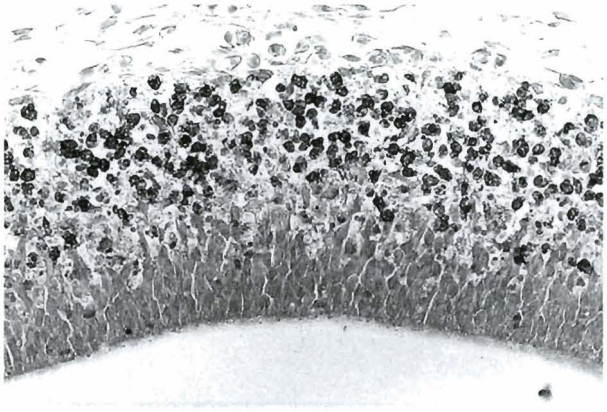

**Fig. 2.** Pyknotic nuclei in the CNS are positively stained by the TUNEL method. 24HAT. x 7,300

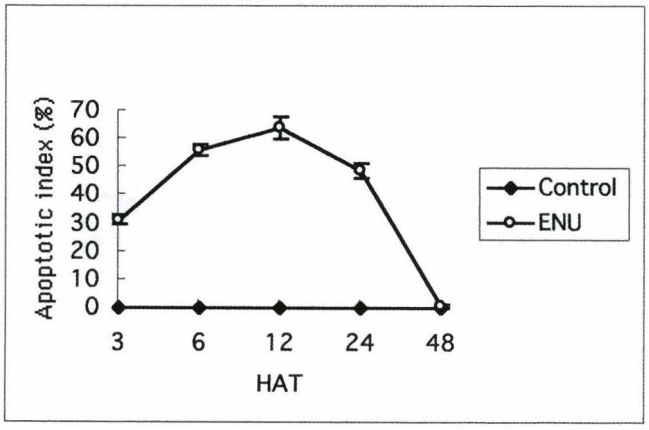


**Fig. 3.** Apoptotic index in the dorsal telencephalic wall of rat fetuses following ENU-administration to dams at GD13. Only a few apoptotic cells are seen in the control fetuses.

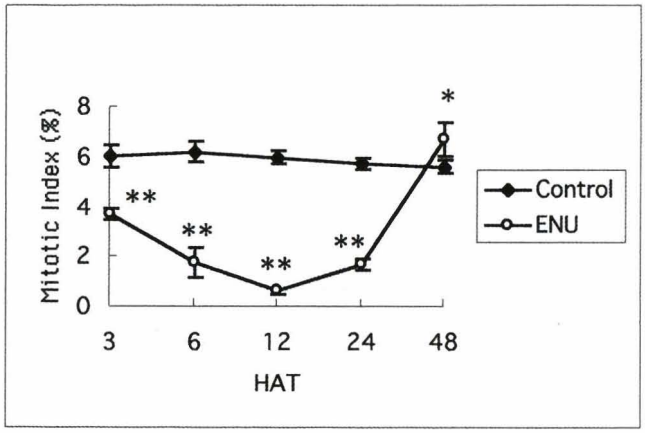


**Fig. 4.** Mitotic index in the dorsal telencephalic wall of rat fetuses following ENU-administration to dams at GD13. *, $p<0.05$, **. $p<0.01$: significantly different from the control group.

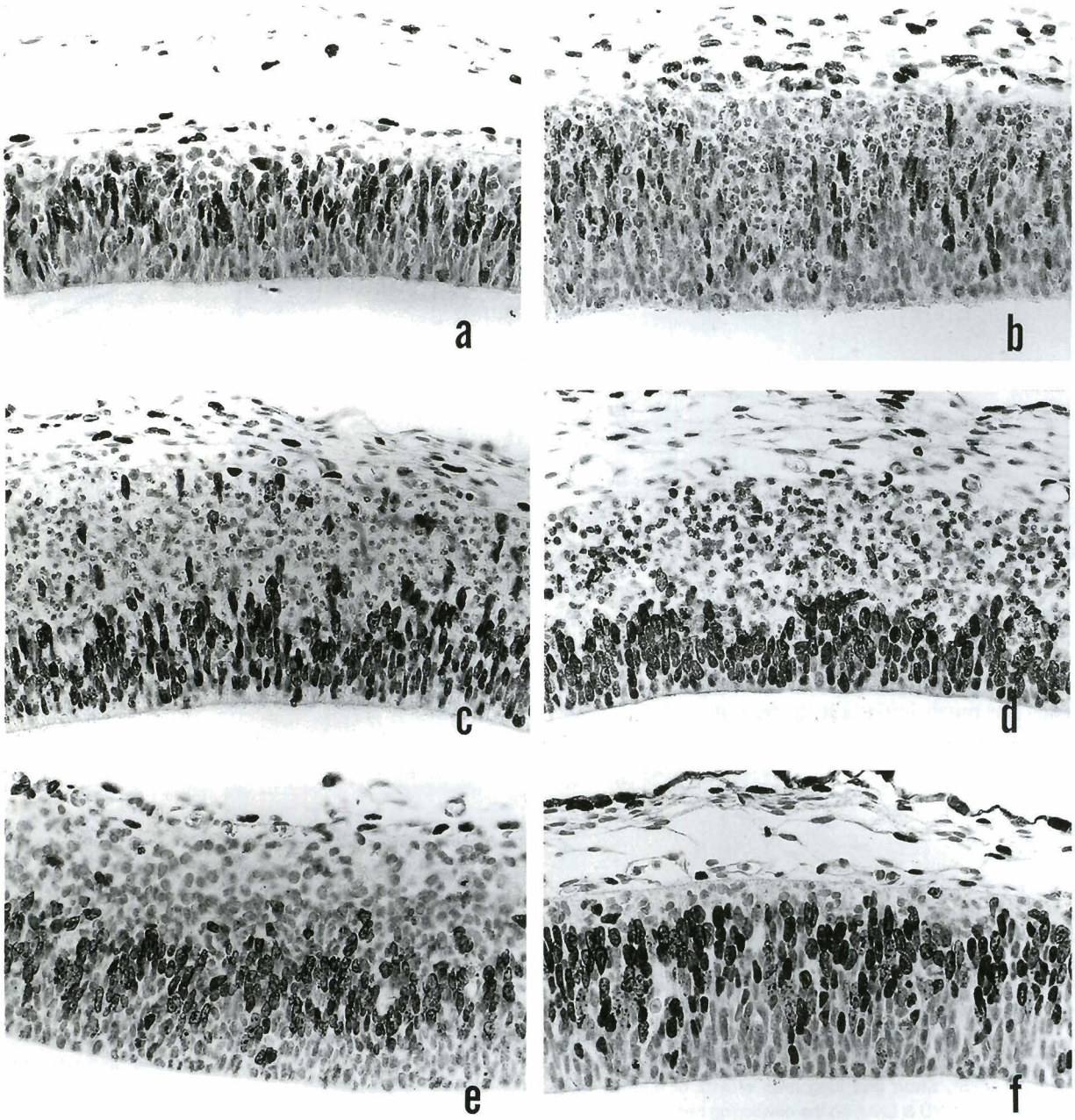


**Fig. 5.** Immunohistochemical staining for BrdU of the dorsal telencephalic wall of the ENU-group at 3 **(a)**, 6 **(b)**, 12 **(c)**, 24 **(d)** and 48 HAT **(e)** and of the control group at 12HAT **(f)**.. The number of BrdU-positive cells gradually decreased from 3 HAT and reached the minimum level at 6 HAT. At 48 HAT, BrdU-labeling index returned to the control level. x 270

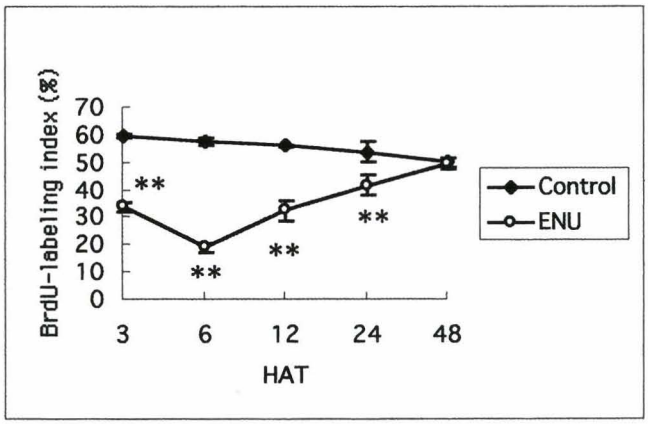


**Fig. 6.** BrdU-labeling index in the dorsal telencephalic wall of rat fetuses following ENU-administration to dams at GD13. *: p<0.05, **: p<0.01: significantly different from the control group.

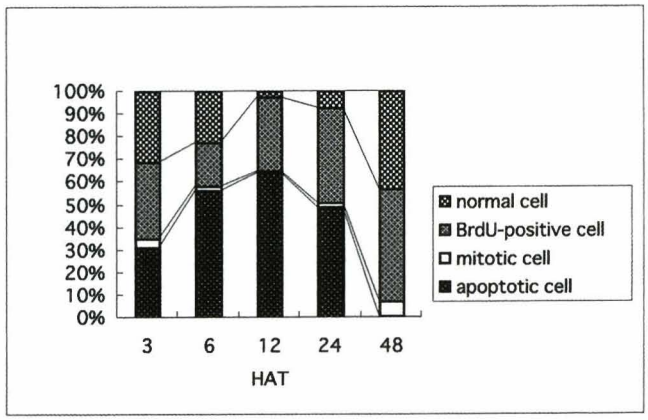


**Fig. 7.** Cell kinetics in the dorsal telencephalic wall of rat fetuses following ENU-administration to dams at GD13.

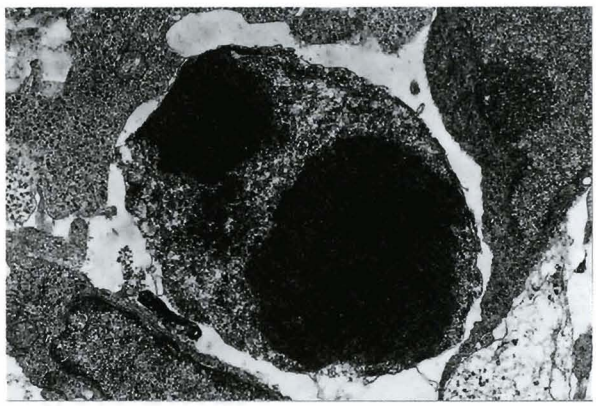

**Fig. 8.** Electron micrograph of pyknotic cells in the CNS. Condensation of nuclear chromatin and fragmentation into small pieces are seen. x 7,500

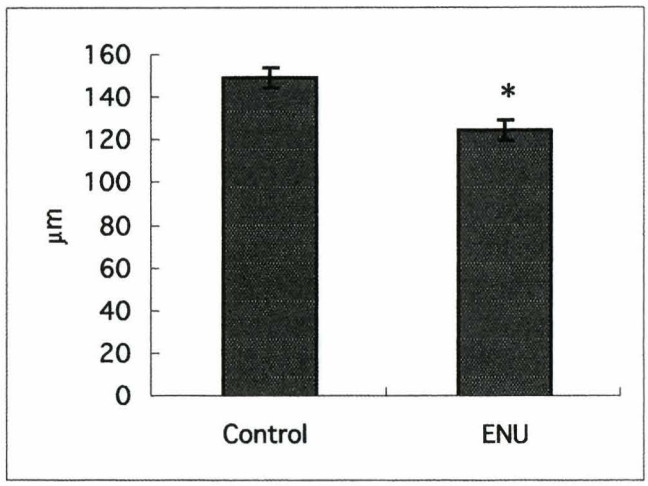


**Fig. 9.** Thickness of the dorsal telencephalic wall at 48 HAT. Thickness of the dorsal telencephalic wall of ENU-group is siginificantly smaller than that of the control group. * p<0.01: significantly different from the control group.

chemicals are much important in these days. This study may provide a clue for elucidating the more detailed mechanisms of fetotoxicity and teratogenicity induced by genotoxic agents.

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