

Hydroxyurea(HU)-induced apoptosis in the mouse fetal tissues

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Summary. Hydroxyurea (HU), a ribonucleotide reductase inhibitor, induces morphological anomalies in the central nervous system (CNS), craniofacial tissues and limb buds in animals, and neonatal respiratory distress in humans. In the present study, pregnant mice were treated with 400 mg/kg of HU at day 13 of gestation, and their fetuses were examined from 1 to 48 hours after treatment (HAT) to find a clue to clarify the mechanisms of HU-induced fetotoxicity and teratogenicity. At 6 and 12 HAT, a moderate to marked increase in the number of pyknotic cells was detected in the CNS and lung. A mild increase in the number of pyknotic cells was also found in the craniofacial mesenchymal tissues, limb buds and so on. These pyknotic cells had nuclei positively stained by the TUNEL method, which is widely used for the detection of apoptotic nuclei, and they also showed electron microscopic characteristics identical to those of apoptotic cells. The present results suggest that the HU-induced fetotoxicity is characterized by excess apoptotic cell death in the fetal tissues, and that such excess cell death in the fetal CNS, lung, craniofacial tissue and limb bud may have a certain relation to the later occurrence of morphological or functional anomalies reported in these tissues following HU-administration.

Key words : Apoptosis, Fetus, Hydroxyurea, Mouse

Introduction

Hydroxyurea (HU), a ribonucleotide reductase inhibitor, is now used for the treatment of various types of cancer, human immunodeficiency virus infection and sickle cell anemia. On the other hand, HU is also known as a teratogen. Since Murphy and Chaube (1964) first reported teratogenicity of HU in rats, there are many

reports of HU-related teratogenic effects in many experimental animal species (Philips et al., 1967; Roll and Baer, 1969; Theisen et al., 1973; Wilson et al., 1975; Aliverti et al., 1980; Barr and Beaudoin, 1981; Desesso et al., 1994). HU induces anomalies in the central nervous system (CNS), craniofacial tissues, skull and limbs (Butcher et al., 1973; Barr and Beaudoin, 1981). In addition, growth retardation was observed in offspring from dams administered with HU in the late stage of pregnancy (Butcher et al., 1973). However, there are only a few reports concerning the mechanisms of HU-induced fetotoxicity and teratogenicity. The number of chemicals with teratogenicity is increasing year by year in the environment surrounding humans and animals, and therefore the mechanisms of fetotoxicity which may be induced by these chemicals are much of interest these days.

This study was carried out to find a clue to clarify the mechanisms of HU-induced fetotoxicity and teratogenicity. The protocol of the present study was approved by the Animal Use and Care Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

Materials and methods

Animals

Thirty-six 8-week-old pregnant mice of Crj:CD-1 (ICR) strain were obtained from Charles River Japan Co., Yokohama, Japan. They were kept in an animal room under controlled conditions (temperature, 23± 2 °C; relative humidity, 55± %) using an isolator caging system (Niki Shoji, Co., Tokyo) and were fed commercial pellets (MF, Oriental Yeast Co., Tokyo) and water *ad libitum*.

Chemical

HU (Sigma, St. Louis, MO) was dissolved in distilled water immediately before the treatment, and the concentration was adjusted to 60 mg/ml.

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Treatments

Thirty pregnant mice were injected with 400mg/kg b.w. of HU intraperitoneally (i.p.) at day 13 of gestation, and 5 dams were sacrificed by heart puncture under ether anesthesia at 1, 3, 6, 12, 24, and 48 hours after treatment (HAT), respectively. The six remaining pregnant mice were injected i.p. with distilled water alone at day 13 of gestation, and two dams were sacrificed in the same way at 3, 12, and 48 HAT, respectively.

Histopathology

Fetuses were collected by Caesarian section and fixed in 10% neutral- buffered formalin. Paraffin sections (4 μ 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. As compared with the control group, the incidence of pyknotic cells in the mouse fetal tissues of the HU-treated group was graded as follows: -, no significant increase; +, mild increase; ++, moderate increase; and +++, marked increase.

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), using a commercial apoptosis detection kit (ApopTag In situ Apoptosis Detection Kit; Oncor, Gaithersburg, MD). 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. TUNEL-positive cells in the CNS were counted under a light microscope (x400). The number of TUNEL-positive cell mm² was expressed as mean \pm standard deviation (SD) of 5 dams (5 fetuses/dam) at each point of examination, and statistical analysis was done by student's *t*-test between the control and HU-treated groups.

Electron microscopy

Small pieces of fetal tissues were fixed in 2% glutaraldehyde in 0.1M phosphate buffer (pH7.4), postfixed in 1% osmium tetroxide in the same buffer, and embedded in epoxy resin (Oken, Shoji Co., Tokyo). 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 Co., Ltd., Tokyo).

Results

There was no significant difference in the body weight gain between the control and HU-treated groups. In addition, neither death nor resorption of fetuses were observed throughout the experimental period.

Histologically, there was no significant difference in the histology of fetuses between the control and HU-treated groups at 1 HAT. As shown in Table 1, the number of pyknotic cells in the CNS began to increase at 3 HAT (Fig. 1a), peaked at 12 HAT (Fig. 1b), and decreased at 24 HAT (Fig. 1c). Pyknotic cells in the CNS were mainly observed in the middle layer of the ventricular zone at 3 HAT (Fig. 1a), and in the middle to dorsal layers at 12 (Fig. 1b) and 24 HAT (Fig. 1c), respectively. Almost all of the nuclei of these pyknotic cells were positively stained by the TUNEL method (Fig. 2), and the sequence of the number of TUNEL-positive neuroepithelial cells corresponded well to that of pyknotic neuroepithelial cells (Fig. 3).

A similar but somewhat less prominent increase in the number of pyknotic cells was observed in mesenchymal cells of the lung (Table 1, Fig. 4a). A small number of bronchial and bronchiolar epithelial cells also showed pyknosis at 6 and 12 HAT. In addition, a mild increase in the number of pyknotic cells was also detected in hematopoietic progenitor cells of the liver (Fig. 4b), epithelial cells of the alimentary tract, and mesenchymal cells of craniofacial tissues (Fig. 4c), kidney (Fig. 4d) and limb bud from 3 to 24 HAT (Table 1). At 48 HAT, there was no significant difference in the histology of fetuses between the control and HU-treated groups (Table 1).

Few pyknotic cells were seen in the fetal tissues of the control group throughout the experimental period (Fig. 1d).

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. Some nuclei were fragmented into small pieces, which were frequently ingested by adjacent cells and/or macrophages (Fig. 5).

Table 1. The incidence of pyknotic cells in the mouse fetal tissues obtained from dams treated with HU at day 13 of gestation.

	1 HAT	3 HAT	6 HAT	12 HAT	24 HAT	48 HAT
CNS	-	+	++	+++	+	-
Lung	-	+	++	++	+	-
Mesenchyme	-	+	+	+	+	-
Liver	-	+	+	+	+	-
Kidney	-	-	+	+	-	-
Alimentary tract	-	-	+	+	-	-

HAT: Hours after treatment; -: no significant increase; +: mild increase; ++: moderate increase; +++: marked increase.

HU-induced apoptosis in mouse fetus

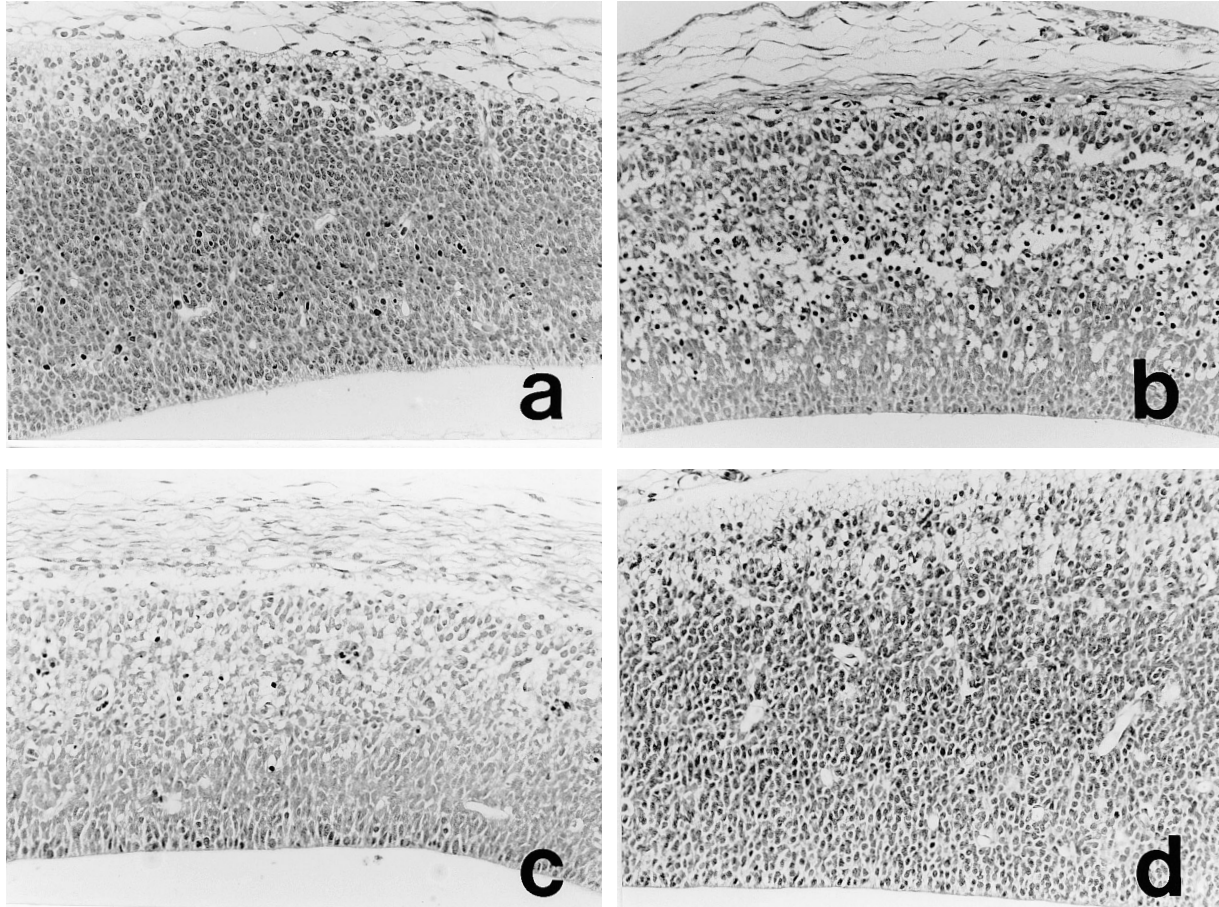


Fig 1. Pyknotic cells in the telencephalic wall of fetuses of the HU-treated group at 3 HAT (a), 12 HAT (b), 24 HAT (c), and of a fetus of the control group (d). HE, x 300

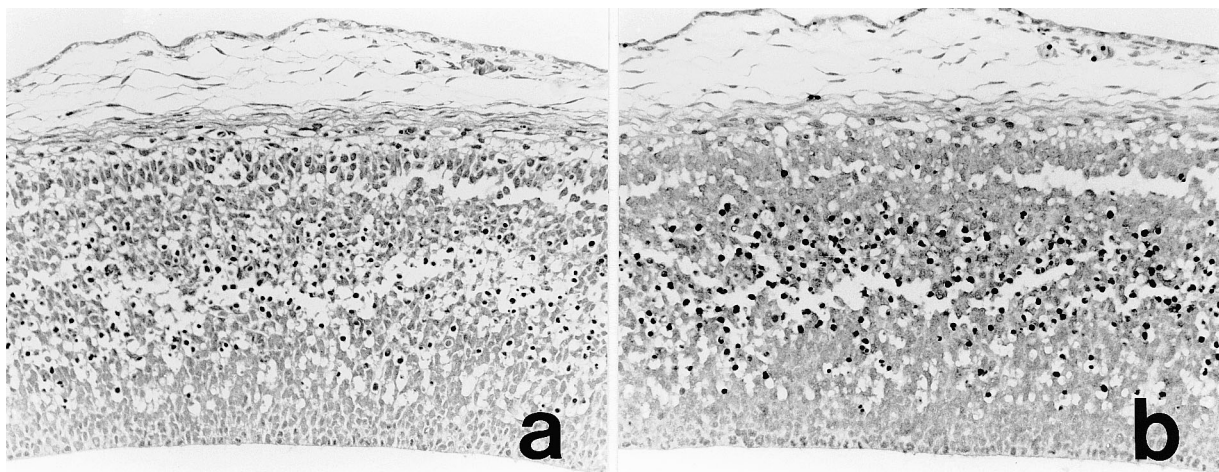


Fig 2. Telencephalic wall of a fetus of the HU-treated group at 12 HAT. Almost all of the pyknotic cells (a, HE stain) are TUNEL-positive (b, immunostaining for TUNEL). Serial sections, x 300

Discussion

HU-induced apoptosis in mouse fetal tissues was examined for up to 48 hours after a single HU-treatment to pregnant mice at day 13 of gestation.

Almost all of the nuclei of pyknotic cells observed in the fetal tissues of the HU-treated groups were positively stained by the TUNEL method which detects fragmented DNA *in situ* and is widely used for the evaluation of apoptotic cells. Although several investigators recently demonstrated that strictly speaking the TUNEL technique is not specific for apoptosis and it also detects a small population of necrotic cells (De Torres et al., 1997; Levin et al., 1999), electron microscopic features of these cells fulfilled the morphological characteristics of apoptotic cells (Ihara et al., 1998; Shinozuka et al., 1998; Ishigami et al., 2001; Katayama et al., 2001; Poapolathep et al., 2002). Therefore, it is reasonable to consider that these pyknotic cells observed in the fetal tissues of the HU-treated group are apoptotic ones.

In the CNS, apoptosis was first seen in the neuroepithelial cells in the middle layer of the ventricular zone at 3 HAT. Thereafter, apoptosis was

observed in the neuroepithelial cells in the middle to dorsal layers of the ventricular zone at 12 and 24 HAT. It is said that the neuroepithelial cells in the dorsal layer

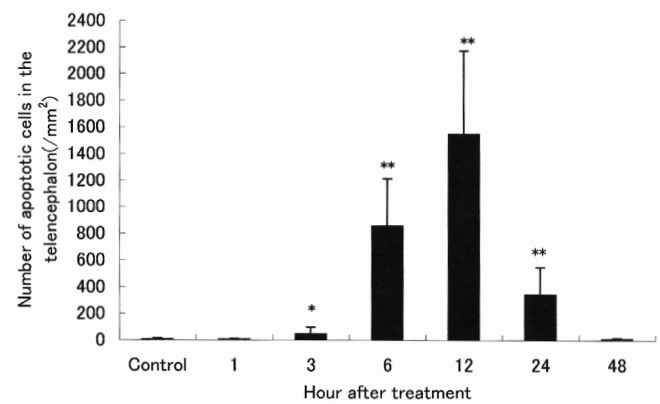


Fig 3. Changes in the number of TUNEL-positive neuroepithelial cells in the telencephalic wall of fetuses of the HU-treated group. Each value represents the mean \pm SD of 5 dams. * $p<0.05$, ** $p<0.01$: Significantly different from the control.

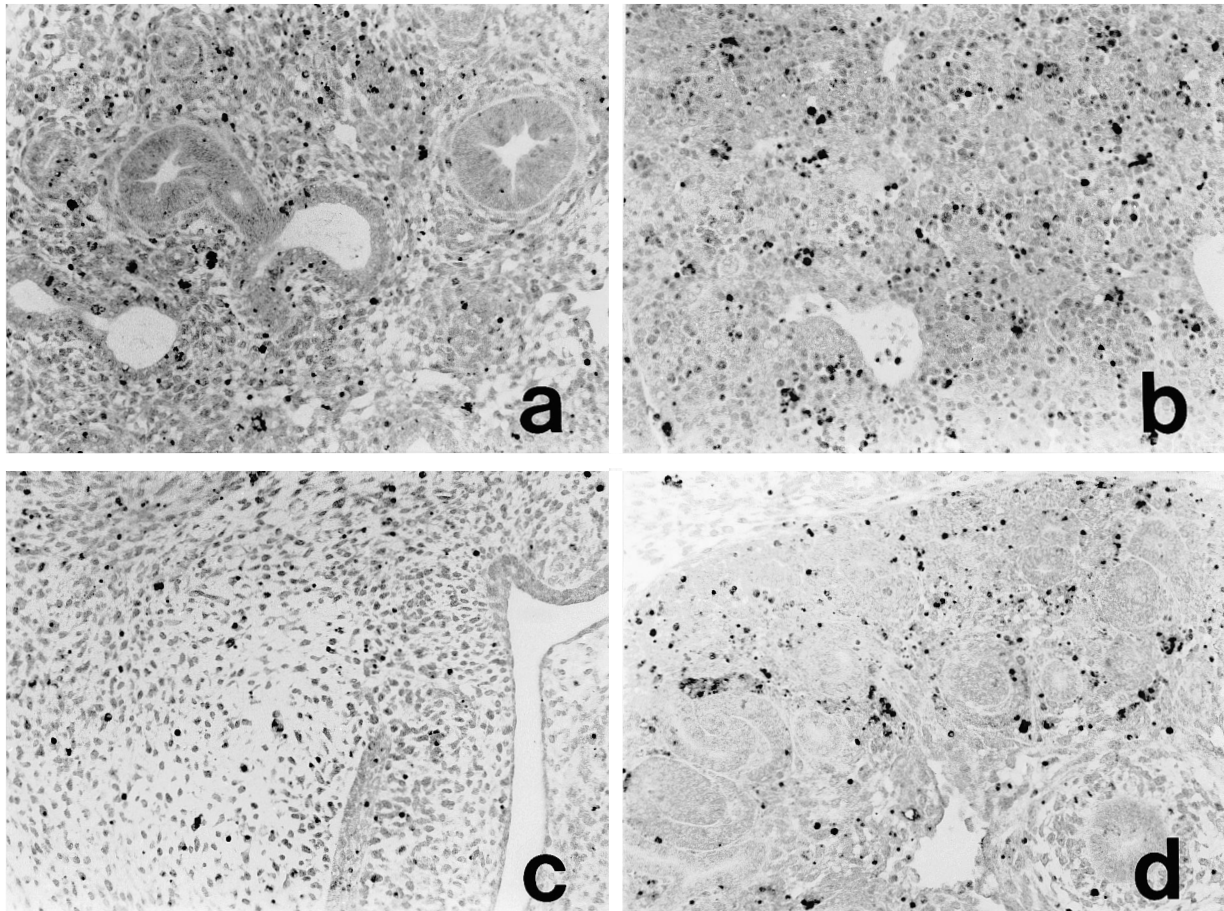


Fig. 4. Pyknotic cells in the lung (a), liver (b), craniofacial tissue (c), and kidney (d) of a fetus of the HU-treated group at 12 HAT. x 300

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actively synthesize DNA (Langman et al., 1966), and those in the middle layer are in the G1 or G2 phase. It is also said that HU inhibits DNA synthesis through its ribonucleotide reductase inhibiting effect (Yarbro, 1992). HU has also been known to cause apoptosis in the CNS in the mouse embryo culture (Zucker et al., 1998).

Enhancement of apoptotic cell death in the fetal CNS was also reported in rats and/or mice following administration of 5-azacytidine, a DNA hypomethylating agent (Lu et al., 1998; Ueno et al., 2002), of ethylnitrosourea (ENU), a DNA alkylating agent (Katayama et al., 2001, 2002), of T-2 toxin, a type of trichothecene mycotoxin having an inhibitory effect on DNA synthesis, and of γ -radiation (Borovitskaya et al., 1996). From these findings, the fetal CNS seems to be highly sensitive to genotoxic stimuli and develops apoptosis easily.

In the fetal CNS of the HU-treated group, the number of apoptotic neuroepithelial cells began to increase at 3 HAT, peaked at 12 HAT, decreased at 24 HAT, and returned to the control level at 48 HAT. The same peak time of the number of apoptotic cells (12 HAT) was also observed in the fetal CNS after treatment with ENU (Katayama et al., 2001), 5-azacytidine (Ueno et al., 2002) and T-2 toxin (Ishigami et al., 2001), but the time of initiation and/or termination of apoptosis differed from each other. Such differences in the time course of apoptotic responses may reflect the differences in pharmacokinetics, metabolic activation and signaling pathways involved in apoptosis among chemicals. Further studies on the mechanisms of HU-induced apoptosis in the fetal CNS are now in progress.

Similar development of apoptosis was detected in

the fetal lung of the HU-treated group, although it was somewhat less prominent than in the fetal CNS. Apoptosis was mainly observed in mesenchymal cells, and a small number of bronchial and bronchiolar epithelial cells were also involved. In humans, neonatal respiratory distress has been reported in infants from women exposed to HU during pregnancy (Thauvin-Robinet et al., 2001), and this may have a certain relation to the above-mentioned HU-induced toxicity in the fetal lung.

In addition to the CNS and lung, mild enhancement of apoptotic cell death was also observed in fetal tissues such as mesenchymal cells in the kidney, craniofacial tissues and limb buds, epithelial cells of the alimentary tract, and hematopoietic progenitor cells in the liver after HU-treatment to dams at day 13 of gestation. On the other hand, anomalies such as hydrocephalus, craniofacial dysgenesis, and ectrodactyly were frequently observed in the offspring following HU administration to their dams (Butcher et al., 1973; Aliverti et al., 1980; Barr and Beaudoin, 1981). These findings suggest that excess cell death by apoptosis in the fetal CNS, craniofacial mesenchymes and limb buds may bring about lack of cell populations required for the later normal histogenesis and organogenesis, resulting in anomalies in these tissues. To clarify this point, further studies on neonates from dams treated with HU are now in progress. In addition, it is important to examine the possibility that HU-induced fetotoxicity may be modified by maternal conditions, because it is reported that HU inhibits cellular and developmental activities in the decidualized and pregnant uteri of rats (Spencer et al., 2000).

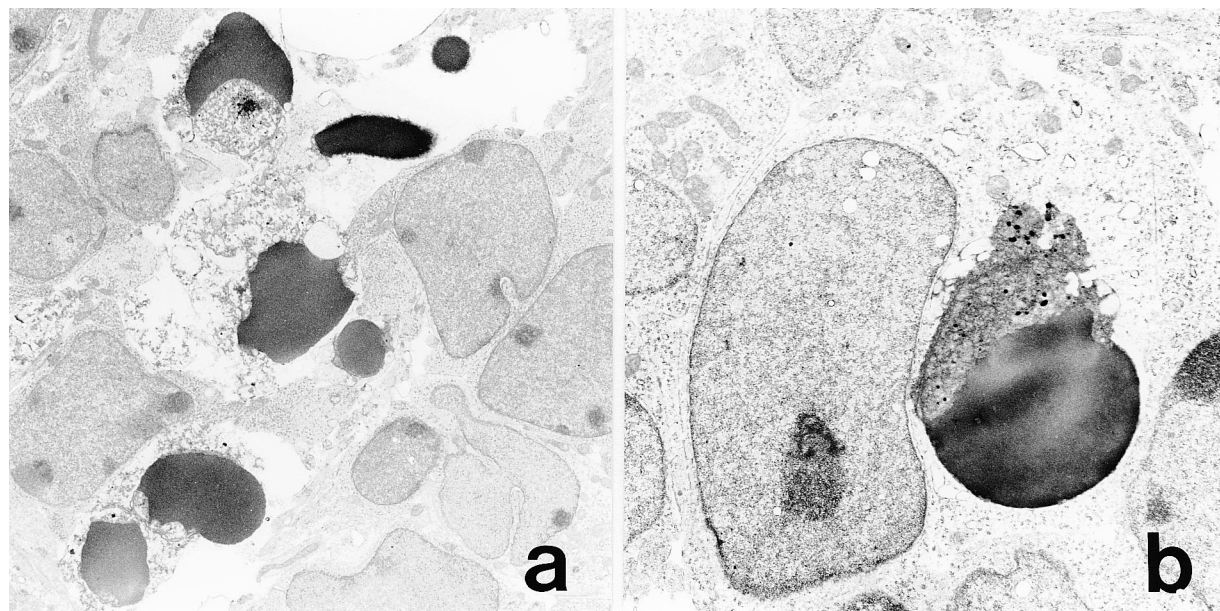


Fig. 5. Electron micrograph of pyknotic cells in the fetal CNS. Condensation of nuclear chromatin and fragmentation into small pieces (**a**) and an apoptotic body ingested by an adjacent cell (**b**). a, x 3,750; b, x 7,500

The results of the present study may provide a clue for investigating the mechanisms of HU-induced fetotoxicity and teratogenicity.

Acknowledgements. We would like to express our thanks to Mr Akira Yasoshima, Department of Veterinary Pathology, the University of Tokyo, for his technical assistance.

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Accepted November 27, 2002