

Original

## ***N,N'*-Bis(2-chloroethyl)-*N*-nitrosourea (BCNU)-induced Apoptosis of Neural Progenitor Cells in the Developing Fetal Rat Brain**

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**Abstract:** *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) is one of the major drugs used in chemotherapy against malignant gliomas due to its effects, such as induction of bifunctional alkylation of DNA and formation of interstrand DNA cross-linkages, and induces cortical malformations in the fetal and neonatal rat brain. In this study, pregnant rats were treated with 7.5 mg/kg of BCNU on gestational day 13 (GD 13), and their fetuses were collected from 12 to 72 hours after BCNU treatment in order to examine the timecourses of morphological and immunohistochemical changes in neural progenitor cells in the developing brain. The number of pyknotic cells in the telencephalon peaked at 24 h and then gradually decreased until 72 h. The majority of these pyknotic cells were positive for cleaved caspase-3, a key executioner of apoptosis. The pyknotic cells showed the ultrastructural characteristics of apoptosis. The number of p53-positive cells began to increase prior to the appearance of apoptotic cells and p21-positive cells. The number of phosphorylated-histone H3-positive cells (mitotic cells) decreased from 24 to 36 h. The number of Iba1-positive cells (microglial cells) in the telencephalon increased from 12 to 48 h. These results suggest that BCNU induces p53-dependent apoptosis and reduces proliferative activity, resulting in reduction of the weight of the telencephalon and the thickness of the telencephalic wall in the fetal brain. This study will help to clarify the mechanisms of BCNU-induced fetal brain toxicity. (*J Toxicol Pathol* 2010; 23: 25–30)

**Key words:** apoptosis, BCNU, neural progenitor cells, rat, fetal brain

### **Introduction**

*N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU), also called carmustine, is widely used in chemotherapy against malignant gliomas<sup>1,2</sup> due to its effects, such as induction of bifunctional alkylation of DNA and formation of interstrand DNA cross-linkages<sup>3,4</sup>.

In general, it is known that neural progenitor cells, which are mainly located in the ventricular zone, proliferate in the fetal developing brain. Thereafter, the neural progenitor cells differentiate into neural cells, i.e., neurons, astrocytes and oligodendrocytes<sup>5</sup>.

It has also been also reported that BCNU induces cortical malformations, such as reduced cortical size, laminar disorganization and heterotopic clusters of neurons, in the fetal and neonatal brain when pregnant rats are

exposed to BCNU<sup>6</sup>. Since such BCNU-induced cortical malformations in the rat brain show similar morphological features to those of cortical dysplasia (CD) in humans with epilepsy<sup>5</sup>, BCNU-induced cortical malformations in rats have been used to study human CD<sup>7</sup>. However, the mechanisms of neural progenitor cell death in the fetal rat brain after BCNU administration to dams are not fully understood.

In order to clarify this, the present study was carried out to examine the progression of neural progenitor cell death in fetal rat brains obtained from BCNU-treated pregnant dams.

### **Material and Methods**

#### *Animals*

Thirty 13-week-old specific pathogen-free pregnant rats of the CrI:CD(SD) strain were obtained from Charles River Laboratories Japan Inc. (Kanagawa, Japan). The animals were individually housed in wire-mesh cages in an air-conditioned animal room (temperature, 23 ± 3°C; relative humidity, 50 ± 20%; ventilation, 12 to 17 /h; lighting, 12h/12h-light/dark cycle) and were given a pelleted diet (CR-

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LPF, Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*.

### Chemical

BCNU (Sigma-Aldrich Corporation, St. Louis, MO, USA) dissolved in 5% glucose solution (Otsuka Pharmaceutical Factory, Tokushima, Japan) was used.

### Experimental design

On gestational day 13 (GD13), 15 pregnant rats were injected i.p. with 7.5 mg/kg of BCNU, and 3 dams were sacrificed by exsanguination from the abdominal aorta under ether anesthesia at 12, 24, 36, 48 or 72 hours after BCNU-treatment. Fetuses were collected from each dam by Caesarean section. The remaining 15 pregnant rats were injected i.p. with 5% glucose solution on GD13, sacrificed in the same way and used as controls. The protocol of the present experiment was conducted according to the Guidelines for Animal Experimentation outlined by the Japanese Association for Laboratory Animal Science (1987).

### Histopathology

The telencephalons of 3 randomly selected fetuses from each dam, 9 fetuses of each time point, were resected and weighed regardless of sex. Due to the minimum size of the telencephalon for histological preparation, the whole bodies of other fetuses were fixed in 10% neutral-buffered formalin, and 2  $\mu\text{m}$  longitudinal paraffin sections were stained with hematoxylin and eosin (HE). For further histological analysis, 3 fetal specimens in which we could recognize the telencephalic vesicle were selected from each dam (9 telencephalons for each time point).

### Immunohistochemistry

For immunohistochemical detection of cleaved caspase-3 (Asp175), p53, p21, phosphorylated-histone H3 (Ser10) and Iba1, paraffin-embedded sections were deparaffinized and immersed in 10 mM citrate buffer at pH 6.0 and heated at 121°C for 15 min by autoclaving. After being washed in Tris-buffered saline (TBS) containing 50 mM Tris-HCl (pH 7.6) and 150 mM NaCl, the sections were placed in 3% H<sub>2</sub>O<sub>2</sub>-containing TBS for 5 min to inactivate endogenous peroxidases. The sections were then incubated in 8% skimmed milk at 37°C for 30 min and reacted with rabbit anti-cleaved caspase-3 antibody (dilution, 1:400; Cell Signaling Technology, Beverly, MA, USA), rabbit anti-p53 polyclonal antibody (dilution, 1:300; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-p21 monoclonal antibody (dilution, 1:25; Dako Cytomation, Carpinteria, CA, USA), rabbit anti-phosphorylated-histone H3 (Ser10) polyclonal antibody (dilution, 1:100; Cell Signaling Technology) or rabbit anti-Iba1 polyclonal antibody (dilution, 1:250; Wako, Osaka, Japan) at 4°C overnight. The sections were then reacted with EnVision+system-peroxidase labeled polymer conjugated to anti-rabbit IgG (DAKO) or to anti-mouse IgG (DAKO) at 37°C for 40 min. Positive signals were visualized by the

peroxidase-diaminobenzidine (DAB) method. The sections were then counterstained with methyl green.

### Electron microscopy

Small pieces of the telencephalon of one fetus 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 epoxy resin (Oken Shoji, Tokyo, Japan). Ultrathin sections were stained with toluidine blue for observation under a light microscope. Ultrathin sections of the selected areas were double-stained with uranyl acetate and lead citrate and observed under a JEM-100CX II transmission electron microscope (Nippon Denshi, Tokyo, Japan).

### Morphometry

The thickness of the telencephalic wall recognized as the thickest distance between the telencephalic vesicle and surface was measured in HE-stained sections by micrometer under a light microscope in the ventricular zone (VZ). Pyknotic cells were counted in the same way on HE-stained sections under a light microscope ( $\times 400$ ). Three hundred cells were counted in each fetus. The cleaved caspase-3-, p53-, p21-, phosphorylated-histone H3- and Iba1-positive cells in the immunostained sections were counted in the same way. In addition, the microglial cell index (the number of Iba1-positive cells/100  $\mu\text{m}^2$ ) was represented as the mean of 3 fetuses/dam  $\times$  3 dams.

### Statistical analysis

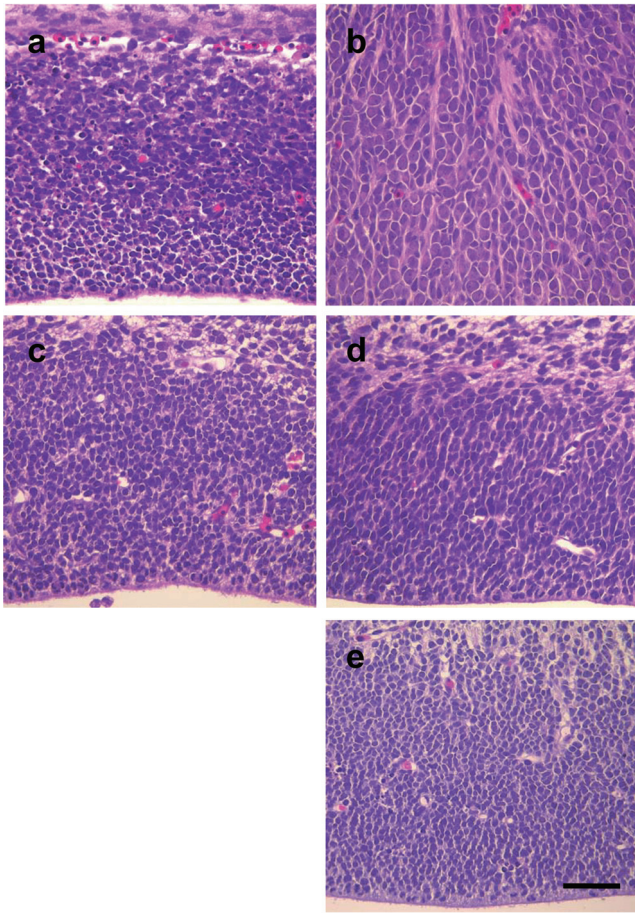
The weight of the telencephalon, thickness of the telencephalic wall and percentages of pyknotic cells and cleaved caspase-3-, p53-, p21-, phosphorylated-histone H3- and Iba1-positive cells in the BCNU-treated groups were expressed as the mean  $\pm$  standard deviation (SD) at each point of examination. Statistical analysis was carried out using the Student's *t*-test.

## Results

In the fetal brains obtained from dams exposed to BCNU on GD13, pyknosis of neural progenitor cells was most prominent in the telencephalon (Fig. 1a), although it was also observed in the diencephalon (Fig. 1b), mesencephalon (Fig. 1c), metencephalon (Fig. 1d) and spinal cord (Fig. 1e). Therefore, we focused on the telencephalon to investigate the progression of BCNU-induced changes in the developing brain. On the other hand, pyknosis of neural progenitor cell was not observed in the control groups.

The weight of the telencephalon and thickness of the telencephalic wall in the BCNU-treated fetuses initially showed significant reductions compared with those in the control fetuses (Fig. 2).

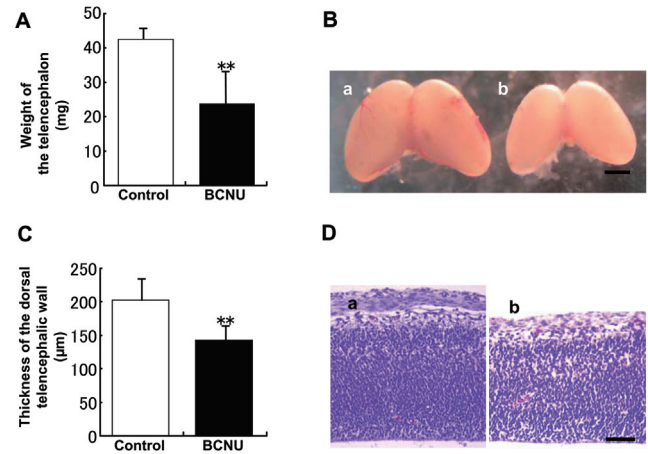
In the telencephalon of the BCNU-treated fetuses, the number of pyknotic neural progenitor cells peaked at 24 h (Fig. 3Ab), gradually decreased toward 48 h and returned to



**Fig. 1.** Histology of the telencephalon (a), diencephalon (b), mesencephalon (c), metencephalon (d) and spinal cord (e) of BCNU-treated fetuses at 24 h. HE, Bar: 40  $\mu$ m.

the control level by 72 h (Fig. 3B). Pyknotic cells showed a diffuse distribution within the VZ. The number of cleaved caspase-3-positive neural progenitor cells peaked at 24 h (Fig. 4Ab) and returned to the control level by 72 h (Fig. 4B). Ultrastructurally, the pyknotic cells were characterized by shrinkage of the cell body and condensation and/or marginalization of nuclear chromatin (Fig. 5). The number of p53-positive neural progenitor cells began to increase at 12 h, peaked at 24 h (Fig. 6Ab) and returned to the control level by 72 h (Fig. 6B). The number of p21-positive neural progenitor cells began to increase at 24 h (Fig. 7Ab), and returned to the control level at 48 h (Fig. 7B). The number of phosphorylated-histone H3 (Ser10)-positive neural progenitor cells (mitotic cells) along the ventricular surface began to decrease at 24 h (Fig. 8Ab), reached the lowest level at 36 h and returned to the control level by 48 h (Fig. 8B). The number of Iba1-positive cells (microglial cells) peaked at 48 h (Fig. 9Ab) and returned to the control level by 72 h (Fig. 9B).

On the other hand, in the telencephalon of the control fetuses, pyknotic, cleaved caspase-3, p53-, p21- and Iba1-positive cells were not detected throughout the experimental period (Figs. 3B, 4B, 6B, 7B and 9B). The number of



**Fig. 2.** A. Weight of the telencephalon of the fetal brain at 72 h. B. The telencephalon of a control fetus (a) and that of a BCNU-treated fetus (b) at 72 h (HE). C. Thickness of the telencephalic wall at 72 h. D. The telencephalic wall of a control fetus (a) and that of a BCNU-treated fetus (b) at 72 h (HE). Each value represents the mean  $\pm$  SD of 3 randomly chosen fetuses per dam ( $\times$  3 dams). \*\* $P < 0.01$ . Bar: B, 1 mm; D, 30  $\mu$ m.

phosphorylated-histone H3-positive mitotic cells was significantly higher in the control fetuses than in the BCNU-treated fetuses from 12 to 36 h (Fig. 8B).

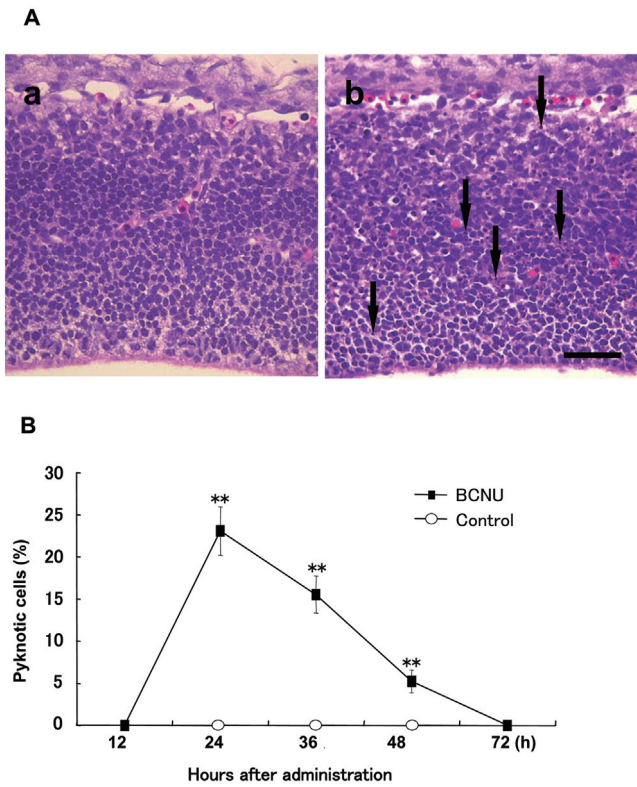
## Discussion

In the present study, neural progenitor cell death was sequentially examined in the telencephalon of the fetal rat brain following BCNU-administration to their dams on GD13.

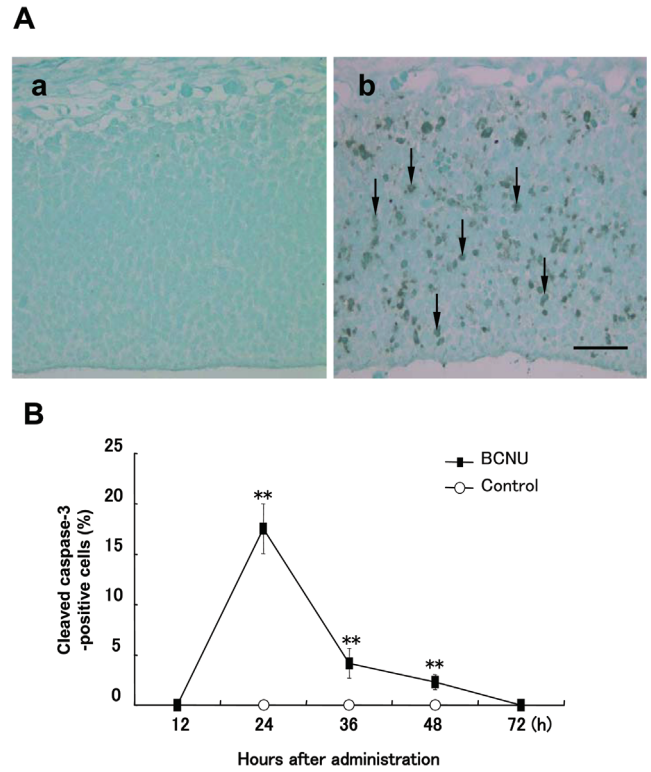
The majority of pyknotic cells observed in the present study were positive for cleaved caspase-3. Activated caspase-3 is a key executioner of apoptosis, and activation of caspase-3 in neural progenitor cells was observed during the process of DNA-damaging agent-induced fetal brain damage<sup>8,9</sup> as well as during normal fetal brain development. In addition, the pyknotic cells showed the ultrastructural characteristics of apoptotic cells<sup>10</sup>. These findings indicate that BCNU induces apoptotic cell death in neural progenitor cells in the fetal rat brain.

In the present study, prior to the appearance of apoptotic cells and p21-positive cells, the number of p53-positive neural progenitor cells showed a significant increase at 12 h. p53 is a guardian of the genome, and it transactivates p21 and other target genes, resulting in apoptotic cell death and cell cycle arrest<sup>11</sup>. Transactivated p21 is known to inhibit cyclin dependent kinase activity, resulting in depression of the proliferative activity of cells<sup>12,13</sup>. Judging from these findings, it is reasonable to consider that BCNU-induced neural progenitor cell apoptosis may be a p53-related phenomenon.

Increased apoptosis of neural progenitor cells has also been reported in fetal rat and mouse brains after treatment of



**Fig. 3.** A. Histology of the telencephalic wall of a control fetus (a) and that of a BCNU-treated fetus (b) at 24 h. B. Pyknotic cell index (%) in the fetal telencephalic wall. Each value represents the mean  $\pm$  SD of 3 randomly chosen fetuses per dam ( $\times$  3 dams). \*\* $P < 0.01$ . Arrows: pyknotic cells. HE, Bar: 40  $\mu$ m.



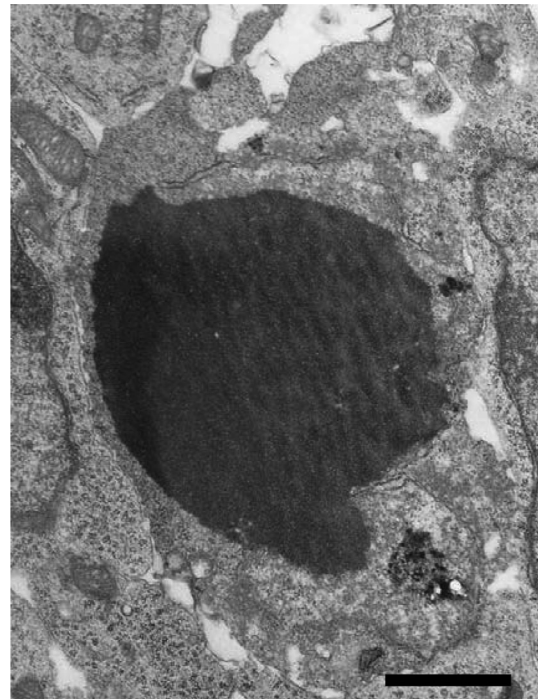
**Fig. 4.** A. Immunostaining for cleaved caspase-3 in the telencephalic wall of a control fetus (a) and that of a BCNU-treated fetus (b) at 24 h. B: Cleaved caspase-3-positive cell index (%) in the fetal telencephalic wall. \*\* $P < 0.01$ . Arrows: cleaved caspase-3-positive cells. Bar: 30  $\mu$ m.

their dams with DNA damaging agents such as 5-azacytidine<sup>14, 15</sup>, ethylnitrosourea<sup>16</sup>, etoposide<sup>17</sup>, hydroxyurea<sup>18</sup>, 1- $\beta$ -D-arabinofuranosylcytosine<sup>19</sup> and 6-mercaptopurine<sup>20</sup>. Although the apoptosis of neural progenitor cells in the fetal brain induced by these agents are all considered to be p53-dependent, the peak time of apoptosis differs among them.

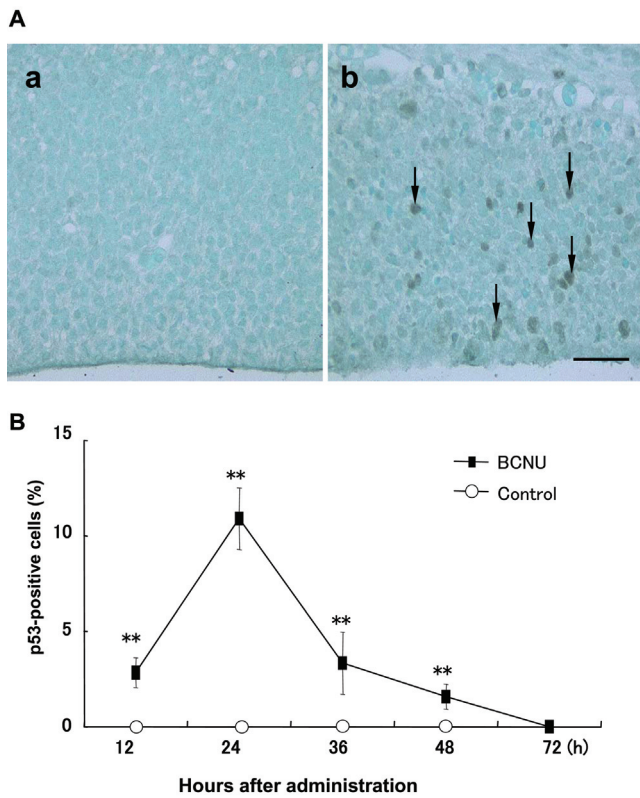
It is well known that histone H3 protein is phosphorylated during mitosis at two serine residues, Ser10 and Ser28<sup>21</sup>. In the present BCNU-treated fetal rat brain, the number of phosphorylated-histone H3 (Ser10)-positive cells along the ventricular surface began to decrease at 24 h, reached the lowest level at 36 h and recovered to the control level by 48 h. This suggests that BCNU induces depression of proliferative activity in neural progenitor cells during apoptosis.

In the present study, the weight of the telencephalon and thickness of the telencephalic wall were significantly decreased at 72 h in the BCNU-treated fetuses. This was thought to be a consequence of the above-mentioned BCNU-induced apoptosis, depression of proliferative activity and blockade of mitosis in neural progenitor cells.

Incidentally, Iba1 is widely used as a marker of microglial cells<sup>22</sup>. In the present study, no Iba1-positive cells were detected in the telencephalic wall of the control



**Fig. 5.** Electron microscopy of a neural progenitor cell in the telencephalic wall of a BCNU-treated fetus at 24 h. Condensation of nuclear chromatin. Bar: 3  $\mu$ m.



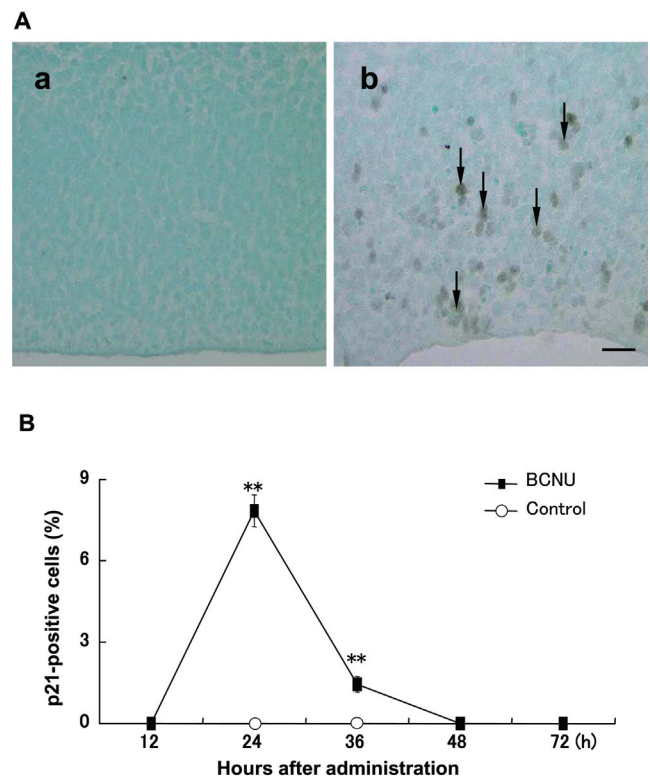
**Fig. 6.** A. Immunostaining for p53 in the telencephalic wall of a control fetus (a) and that of a BCNU-treated fetus (b) at 24 h. B. p53-positive cell index (%) in the fetal telencephalic wall. \*\* $P < 0.01$ . Arrows: p53-positive cells. Bar: 40  $\mu\text{m}$ .

fetal brain throughout the experimental period, while the number of Iba1-positive cells increased from 12 to 48 h in the telencephalic wall of the BCNU-treated fetal brains. This suggests that microglial cells migrate into the telencephalic wall to ingest and remove apoptotic neural progenitor cells.

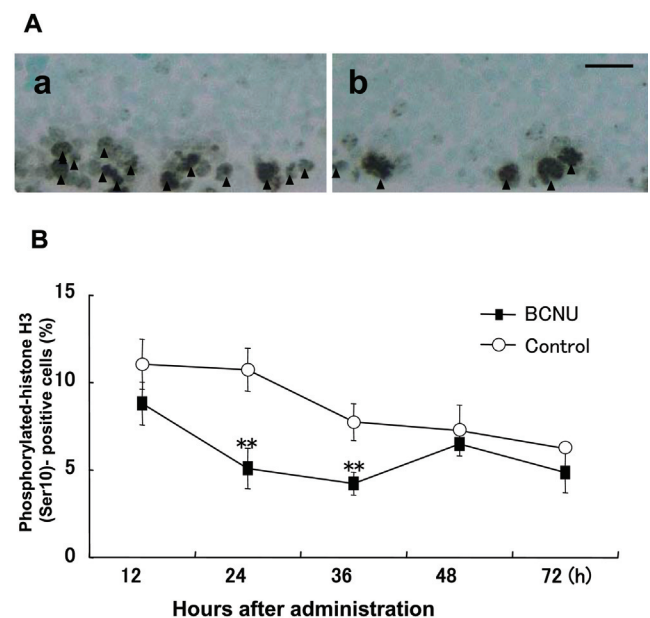
In conclusion, the present study clarified that BCNU induces p53-dependent apoptotic cell death and depresses cell proliferative activity in the fetal rat brain. These effects may cause development of BCNU-induced fetal and neonatal cortical malformations<sup>5</sup>. The present results will help to clarify the mechanisms of BCNU-induced fetal brain damage. Further studies on gene expression levels are necessary to elucidate the molecular pathway of BCNU-induced fetal brain toxicity.

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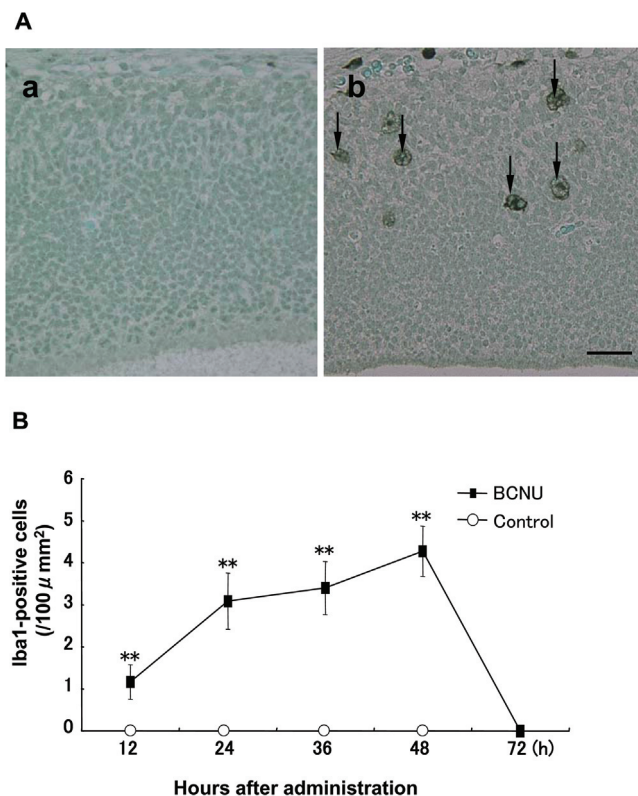
The authors thank Dr. Kunio Doi, Emeritus Professor of the University of Tokyo, for his encouragement during the study and Dr. Rie Andoh of Bozo Research Center Inc., Japan, for her skilled technical assistance during the electron microscopy.



**Fig. 7.** A. Immunostaining for p21 in the telencephalic wall of a control fetus (a) and that of a BCNU-treated fetus (b) at 24 h. B. p21-positive cell index (%) in the fetal telencephalic wall. \*\* $P < 0.01$ . Arrows: p21-positive cells. Bar: 40  $\mu\text{m}$ .



**Fig. 8.** A. Immunostaining for phosphorylated-histone H3 (Ser10) in the ventricular surface of the telencephalic wall of a control fetus (a) and that of a BCNU-treated fetus (b) at 24 h. B. Phosphorylated-histone H3 (Ser10)-positive cell (mitotic cell) index in the fetal telencephalic wall. \*\* $P < 0.01$ . Arrowheads: phosphorylated-histone H3 (Ser10)-positive cells. HE, Bar: 20  $\mu\text{m}$ .



**Fig. 9.** A. Immunostaining for Iba1 in the telencephalic wall of a control fetus (a) and that of a BCNU-treated fetus (b) at 48 h. B. Iba1-positive cell index (%) in the telencephalon. \*\* $P < 0.01$ . Arrows: Iba1-positive cells. Bar: 40  $\mu\text{m}$ .

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