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Transient Intrauterine Hypotension: Effect on Newborn Rat Brain

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ABSTRACT

Intrauterine perfusion failure can cause cerebral malformations. We investigated the effect of transient maternal hypotension on newborn rat brain by inducing hypovolemic hypotension for 2.5 h during early embryonic day 7 (E7) or late (E15) gestation in pregnant rats. We found an increase in the number of TUNEL-positive cells within the periventricular germinative matrix in pups subjected to early gestational hypotension and within the cerebral cortex in those subjected to late gestational hypotension in comparison to sham control animals. These results suggest that episodic maternal hypovolemic hypotension may affect the fetal brain, and apoptotic mechanisms may mediate this effect. **(*Pediatr Res* 49: 45–49, 2001)**

Abbreviations:
EH, early hypotension
EC, early control
LH, late hypotension
LC, late control
BP, blood pressure
TUNEL, terminal deoxynucleotidyl-mediated dUTP nick end labeling
PVGZ, periventricular germinative zone

Hypoxia and perfusion failure during pregnancy can be associated with fetal brain malformations (1, 2). A prospective follow-up study of pregnancies complicated by maternal shock or blood loss revealed an increased incidence of polymicrogyria, hydranencephaly, or porencephaly in the infants (3). Transient hypotensive episodes without blood loss, relatively common events during pregnancy, are also considered potentially harmful to the fetus. However, little clinical data exist on their association with brain malformations (3). In particular, the consequences of early gestational (wk 1–16) hypotension have not been investigated. The pathophysiological events leading to fetal malformations following perfusion failure are also unclear: in addition to necrosis, they may include the induction or acceleration of apoptosis. In this experimental study, we investigated the possible consequences of intrauterine transient hypoperfusion on the newborn's brain by producing early and late gestational hypotension in pregnant rats, and examining histology and apoptosis in their pups.

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METHODS

Gestational hypotension. Twelve 3-mo-old pregnant Wistar rats were studied in four groups: EH group (hypotension applied on E7, $n = 3$), LH group (hypotension on E15, $n = 3$), and their corresponding control groups (EC and LC, $n = 3$ each). They were subjected to transient hypotension by femoral artery catheterization. Briefly, rats were anesthetized following an overnight fasting by i.p. 50 mg/kg ketamine (Ketalar®, Eczacibasi, Istanbul, Turkey) and 8 mg/kg xylasine (Rompun®, Bayer, Istanbul, Turkey), put into Fowler position, and left to spontaneous breathing. Rectal temperature was kept constant between 36.5 and 37.5°C by a homeothermic blanket. The femoral artery was dissected by a vertical incision in the femoral region, hooked from the proximal and ligated at the distal end. A 1-mm incision was made in the middle of the artery and a heparinized Silastic 2 F catheter was inserted. The catheter was connected to a mercury manometer by a stopcock. The proximal hook was then released and blood was allowed to fill the catheter and reach the manometer. After an oscillation and stable BP was obtained, approximately 1 mL blood was slowly drawn into a heparinized syringe until 50% reduction in BP was achieved. This syringe was kept at 37°C. The lowered BP level was maintained for 2.5 h. At the end of this period, blood was reinfused and the femoral artery was ligated. Control groups were subjected to the whole procedure except

hypotension. Rats were allowed to complete the normal course of pregnancy.

The study was approved by the Institutional Animal Ethics Committee.

Histopathological examination and TUNEL staining. Within 24 h of delivery, two rat pups from each mother were randomly selected by a technician. They were decapitated, the brain was fixed in 10% buffered formalin, embedded in paraffin, dehydrated through graded alcohol concentrations, and consecutive 5-μm transverse sections passing through the orbital level were taken.

Brain sections were examined with hematoxylin-eosin (H&E) under 40× magnification for cortical architecture, ventricular integrity, and the presence of necrosis and hemorrhage. Thickness of germinal matrices at the thickest area of the periventricular zone, and frontal, parietal, and occipital cortices were measured by a 40× objective and eyepiece with 1-mm grids. Three measurements were made for each region. Values were expressed as mean of frontal, parietal, and occipital cortex measurements ± SD.

DNA fragmentation was evaluated by the TUNEL method (4). Briefly, poly-L-lysine-coated slides were deparaffinized by overnight incubation, hydrated in decreasing concentrations of ethanol, and incubated with proteinase K (20 mg/mL, 15 min, ApopTag Kit; Appligene Oncor, France). Endogenous peroxidase was blocked in 35% hydrogen peroxide (15 min, room temperature). Nick-end labeling was carried out by a digoxygenin-dUTP containing TdT solution. After washing with buffer, peroxidase-conjugated anti-digoxygenin was added and slides were incubated at room temperature for 30 min. Staining was visualized with 0.05% diaminobenzidine. Methyl green was used for background staining. TUNEL-positive cells, identified as brownish cells having intense basophilia and shrinkage, peripheral condensation, and nuclear fragmentation into uniformly dense basophilic masses were detected and counted by two blinded examiners. The average of their counts was recorded.

Antineurofilament and anti-S-100 antibodies (Sigma Chemical Co., St. Louis, MO, U.S.A.), staining neural and glial cells respectively, were used to characterize the cells in the cerebral regions examined. A normal adult intestinal wall biopsy including the myenteric plexus and adult peripheral nerve were used as positive control tissues.

Table 1. *Weight, duration of gestation, and litter size of the rat groups*

Groups	Weight (g) (mean ± SD)	Duration of gestation (days)	Number of pups
EH (n = 3)	206.6 ± 12.5	27.0 ± 0.5	9.6 ± 1.1
EC (n = 3)	201.6 ± 12.5	27.0 ± 1.0	10.0 ± 1.0
LH (n = 3)	202.0 ± 2.6	27.3 ± 1.0	10.3 ± 0.5
LC (n = 3)	221.6 ± 10.4	26.6 ± 0.5	11. ± 1.5

Table 2. *Cortical and periventricular germinative zone thickness: mean of the frontal, parietal, and occipital measurements*

	Thickness (mm) ± SD			
	Periventricular germinative zone		Cortex	
Pups	Right	Left	Right	Left
EH (n = 6)	7.3 ± 0.5	7.0 ± 0.6	46.0 ± 0.6	44.6 ± 1.0
EC (n = 6)	6.8 ± 0.8	6.8 ± 0.7	46.0 ± 0.8	45.5 ± 1.3
LH (n = 6)	7.0 ± 0.8	6.8 ± 0.7	45.8 ± 0.9	46.0 ± 0.6
LC (n = 6)	6.8 ± 0.7	6.6 ± 0.7	45.8 ± 0.7	45.6 ± 0.5

Mann-Whitney U test was used for statistical comparison of the cell counts.

RESULTS

Average weight, duration of gestation and litter size of the groups are given in Table 1. No stillbirths or neonatal deaths occurred in any of the groups.

The thickness of the germinal matrix and mean cortical tissue was similar in control and hypotension groups, and in the right and left hemispheres in each pup ($U = 11.500$, $p = 0.227$; $U = 9.000$, $p = 0.138$) (Table 2). On microscopic evaluation, no alteration of cortical layering, hemorrhage, or inflammatory infiltration was observed. Visible nuclei and nucleoli and intact cytoplasms in cortical cells excluded the occurrence of significant necrosis.

The number of TUNEL-positive cells showing nuclear condensation, blebbing, and fragmentation was significantly higher in the periventricular matrix of the EH group compared with the LH and control groups ($p = 0.01$) (Table 3, Fig. 1*A–D*). On the other hand, the late hypotension group had significantly more TUNEL-positive cells than all other groups in the parietooccipital region, observed in clusters within the cortical plate ($p = 0.005$, Table 4, Fig. 2 *A–D*).

Table 3. *Number of TUNEL-positive cells within the periventricular germinative zone (PVGZ) and cerebral cortex in the EH and EC groups*

	PVGZ (n)		Cerebral cortex (n)					
Pup #	Right	Left	R frontal	R parietal	R occipital	L frontal	L parietal	L occipital
EH1	12	16	1	1	—	1	1	—
EH2	18	15	1	1	—	1	—	1
EH3	19	20	1	—	1	1	1	—
EH4	11	13	2	1	—	1	1	1
EH5	14	11	—	2	1	2	—	1
EH6	10	12	1	1	1	1	1	1
EC1	4	3	2	—	1	1	2	—
EC2	3	2	—	1	1	—	—	3
EC3	4	2	—	1	1	—	—	3
EC4	2	3	1	—	1	1	1	1
EC5	3	3	2	1	—	1	1	1
EC6	2	2	1	1	1	—	—	1

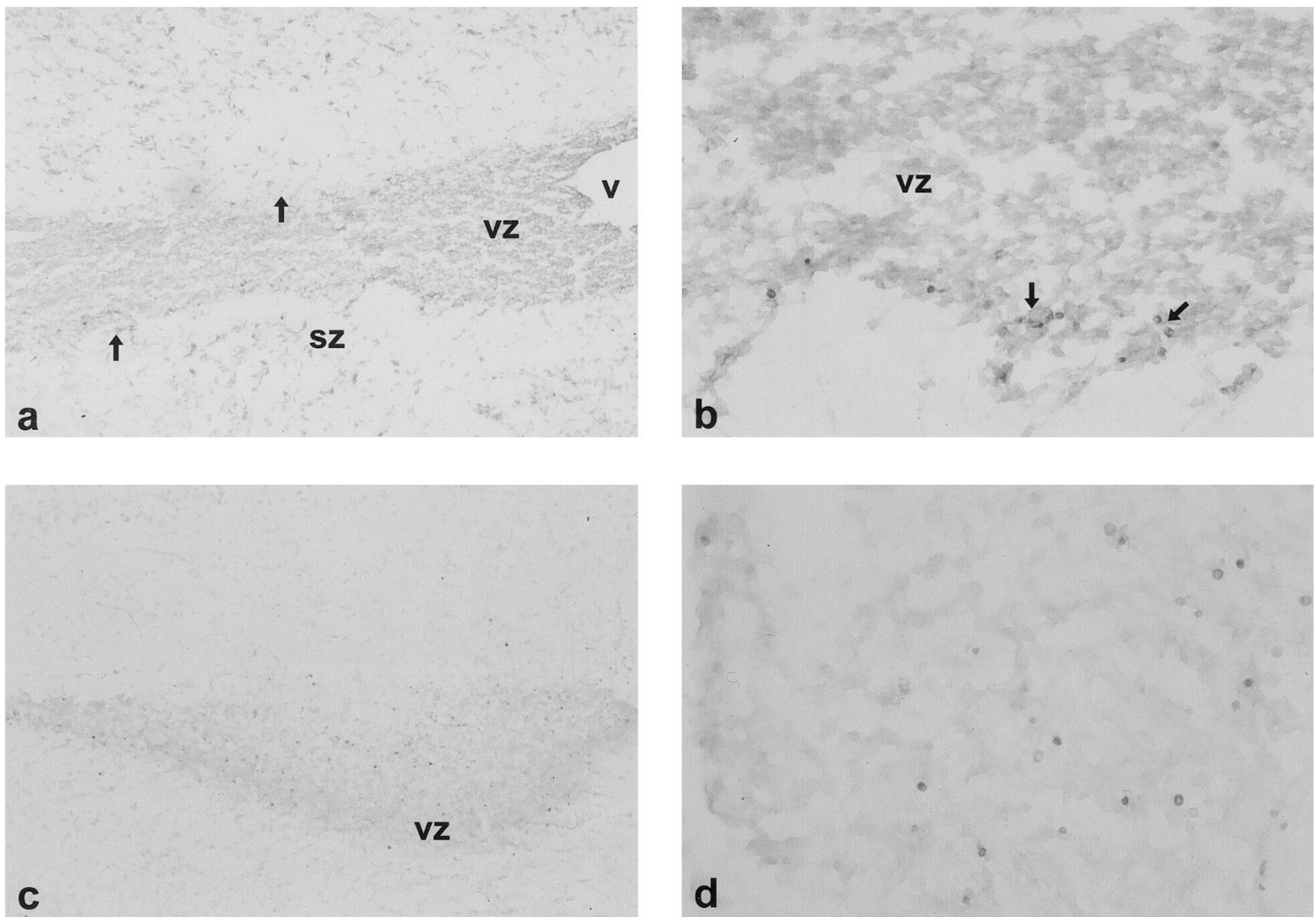

Figure 1. (*A, B*). Periventricular germinative zone, EH group: TUNEL-positive cells (*arrows*) at the border between ventricular (*vz*) and subventricular (*sz*) zones (original magnification ×100 and ×400). *v*, ventricle. (*C, D*) Early control group: the ventricular wall is observed at a tangential section. Fewer apoptotic cells, some with condensed nuclei, are observed at the same location.

Table 4. *Number of TUNEL-positive cells within the periventricular germinative zone (PVGZ) and cerebral cortex in the LH and LC groups*

	PVGZ (*n*)		Cerebral cortex (*n*)					
Pup #	Right	Left	R frontal	R parietal	R occipital	L frontal	L parietal	L occipital
LH1	4	3	9	13	18	8	13	16
LH2	3	3	6	13	14	5	11	15
LH3	3	2	7	12	15	5	12	19
LH4	2	3	7	14	16	6	12	14
LH5	3	2	6	12	14	7	11	14
LH6	2	2	5	14	15	5	12	15
LC1	4	4	1	1	—	1	1	1
LC2	3	2	2	—	1	1	—	1
LC3	2	3	2	—	2	1	1	1
LC4	3	3	1	1	—	1	1	—
LC5	4	3	—	1	1	—	—	2
LC6	3	2	2	—	—	1	—	1

Double immunohistochemistry with antineurofilament and anti-S100 antibodies on TUNEL-stained tissues did not give optimal morphologic results. We therefore examined adjacent sections with TUNEL and immunohistochemistry. The majority (50%–75% in all sections) of periventricular TUNEL-positive cells in the EH group were neurofilament and S-100 negative. Among cortical cells in the LH group, 50% were neurofilament positive and all were S-100 negative. In control tissues stained during the same experiment, the myenteric plexus, endothelial cells, and Schwann cells were positive with these antibodies.

DISCUSSION

Apoptosis has been described in many regions of the vertebrate nervous system including rat cerebral cortex (5). It is a physiologic event starting with DNA fragmentation in early and late periods of development, which is also observed in our

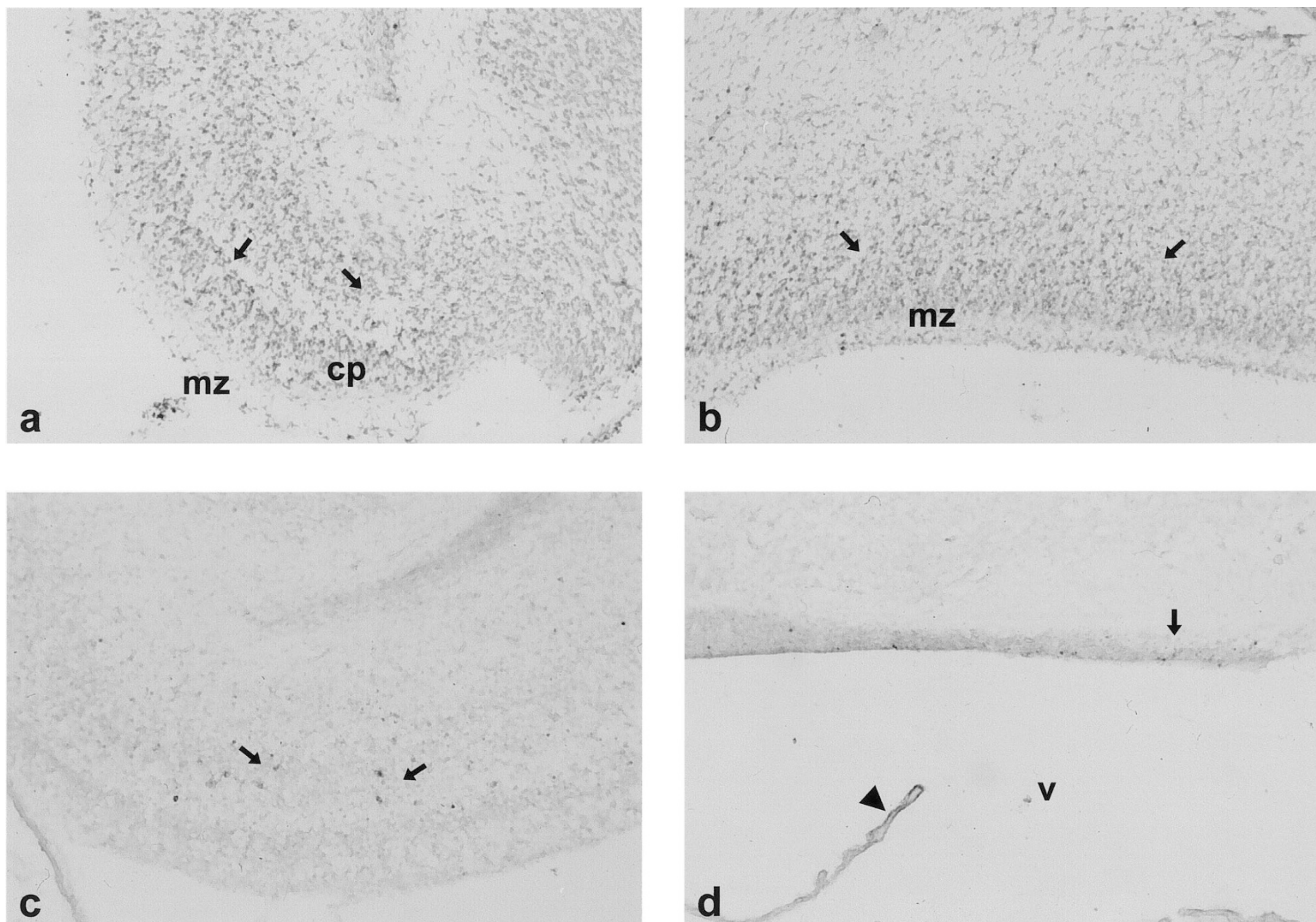

Figure 2. (*A, B*) LH group: numerous TUNEL-positive cells in the occipitoparietal cortex. *mz*, marginal zone; *cp*, cortical plate. (*C, D*) Late control group. Only few TUNEL-positive cells are observed in the (*C*) occipital cortex, and (*D*) ventricular zone. *V*, ventricle, *arrowhead*, choroid plexus. Background stained with methyl green. Original magnification ×100.

control groups as scattered TUNEL-positive cells in the cortex and the periventricular region. On the other hand, rats subjected to intrauterine perfusion failure had markedly increased TUNEL positivity. They showed normal cortical architecture and no major cerebral malformations: this suggests that normal findings on routine histologic examination do not exclude the possibility of cerebral injury.

According to previous studies, transient near-term gestational hypotension may produce edema and necrosis of the fetal brain (6). On the other hand, mid-gestational umbilical cord occlusion in pregnant sheep for 10–20 min, although causing fetal hypotension, bradycardia, acidosis, and hypoxia, produced no cerebral abnormalities (7). These findings illustrate the effect of varying experimental conditions, such as the duration and degree of hypotension, on the type and extent of the damage. Short or mild hypotensive episodes where compensatory mechanisms suffice to maintain tissue perfusion may not produce any morphologic changes. The 2.5-h episode used in our model corresponds to 1 d in human gestation, considered as a clinically significant period. A dose effect is possible in hypotension, as in toxin exposure where different doses of the same agent can cause necrosis or apoptosis (8). Severe maternal hypotension might result in necrosis and major damage in fetal brain, as reported after maternal anaphylaxis, whereas moderate hypotension might only increase the ongoing apoptosis (9). Our model is more likely to reproduce the latter. The timing of the insult is also important: immature neurons are more prone to apoptosis in hypoxia-ischemia (10). Two critical periods have been reported for cerebral effects of fetal perfusion failure: wk 20–24 and the last 10 wk of gestation. Hydranencephalic newborns have a history of perfusion failure around fetal wk 30. Mid-cortical laminar necrosis, cortical overfolding, and polymicrogyria have been associated with perfusion failure after wk 20 of pregnancy. On the other hand, placental insufficiency occurring near birth is known to result in periventricular leukomalacia, multifocal necrosis within the cerebral white matter, or peri- or intraventricular hemorrhage (3).

Apoptosis was originally defined as a morphologic term, and is ideally determined by the appearance of cells under the electron microscope. However, all methods for the detection of apoptosis have limitations, and the difficulty of searching apoptotic cells in large tissue sections by electron microscopy prompt many researchers to use the TUNEL method based on DNA fragmentation (4). As in our samples, morphologic features such as nuclear condensation, blebbing, and apoptotic bodies accompany TUNEL-positivity, and inflammatory changes are absent in this type of cell death.

Difficulties have been reported in the identification of apoptotic cells as neuronal or glial under the light microscope during the developmental period (11). Specific stainings, including *in vivo* injection of cellular markers, may not always provide an answer due to the absence or transient expression of these markers and changes in cell phenotypes during development (12, 13). We interpreted the TUNEL-positive cells observed in the periventricular and cortical regions as neuroblasts and neurons, respectively, based on their size, appearance, localization, and immunostaining, which was negative for periventricular cells, indicating a less differentiated status, and neurofilament positive for 50% of cortical cells, indicating neuronal differentiation. However, the lineage of these cells may not be final yet, as rats on postnatal d 1 correspond to preterm human infants and are still developing organisms (14).

The distribution of the TUNEL-positive cells, *i.e.* periventricular in EH and cortical in LH groups, may result from various factors affecting particular regions of the immature brain such as the type, maturity, developmental activity, and metabolic rate of the target cells. Previous observations showed that apoptosis following insults is more prominent in regions undergoing physiologic cell death (10, 15, 16).

The occurrence of DNA fragmentation 1 wk after the hypotensive episode suggests a late effect of perfusion failure. An interval between the time of the insult and the appearance of apoptosis is observed even in cultured cells deprived of trophic factors. A more protracted process can be expected in the living organism due to the effect of multiple compensatory mechanisms, or because the observed apoptosis is secondary to the death of afferent/efferent cells that has occurred earlier (17). Indeed, apoptotic cells can be detected for days or weeks after the hypoxic-ischemic insult, peaking at 2 wk in adult rat thalamus or for up to 5 d after mechanical trauma to the immature rat brain (16, 18). This may also be the case for our model, and sequential examination of the fetal brain is needed to determine the time course of posthypotension events.

The clinical effects of the findings described in this study are unknown at present. Hypotensive episodes are not uncommon in pregnant women. It generally is speculated that cognitive or behavioral disorders of childhood that are not associated with gross cerebral malformations might be related to microscopical abnormalities of the brain tissue. Investigations of the clinical effects, by allowing these pups to survive and examining their learning tasks and their cerebral structure at later ages, are currently under way.

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