Evaluation of apoptotic cell death following transient maternal hypotension in fetal rat brain: temporal pattern within the first 24 h after procedure

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1. Introduction
Various functional and/or behavioral disorders that manifest throughout life are the result of abnormal intrauterine development of the fetal brain. Although genetic background has an important share, an ‘adverse’ intrauterine environment is a strong modulator of abnormal development (1,2). Among all the prenatal factors, mainly hypoxia–ischemia (HI) and infection are increasingly being recognized as major causes of injury in the developing brain. Low blood pressure and syncopal episodes during pregnancy have been reported to alter fetal perfusion and are associated with increased rates of stillbirth, preterm deliveries, and low birth weight (3–6).

As demonstrated by previous research, perfusion failure during pregnancy causes HI injury to the fetal brain (7–9), which may subsequently result in fetal brain malformations and increased morbidity and mortality (10–12).

Apoptosis plays a prominent role in the development of the nervous system and in the organization of neural circuits in the mature brain (13–16). Furthermore, apoptosis involving caspase-mediated mechanisms plays a role in the susceptibility of the developing brain to HI injury (17–21). The severity and type of neuropathology resulting from HI injury varies depending on the gestational and perinatal age of the infant and the timing and severity of the insult (10,11), as supported by previous observations of the fetal brain on postnatal days 1 and 28 (7,9). Although growing evidence has revealed that adverse in utero experiences are associated with increased risk of neurological, psychological, and psychiatric disorders in later life (11), there is neither a universally accepted therapy nor universally accepted time for the intervention. It is essential to explore all potential and promising targets to prevent or improve the outcome of HI. Since the timing of the injury may affect the outcome and apoptosis has an
important role in shaping the developing brain, it becomes critical to determine the temporal pattern of the apoptotic process in the brain after HI injury following transient maternal systemic hypotension. Based on this background, we aimed to evaluate the early consequences of transient maternal systemic hypotension on apoptosis in the fetal brain during the intrauterine period within the first 24 h after the insult.

2. Materials and methods

2.1. Animals and experimental protocol

Female Sprague–Dawley rats 12 weeks in age (n = 40) were mated with mature males of the same strain and the day on which spermatozoa was observed in the vaginal smears was considered the first day of pregnancy. The animals were randomly allocated into control (n = 20) and hypotension groups (n = 20). They were maintained on a 12-h dark/12-h light illumination sequence (lights were on between 0700 and 1900) at 21 ± 2 °C and 30%–70% relative humidity with ad libitum access to standard pellet rat chow and water. All animal experiments were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Hacettepe University Institutional Ethics Committee for the Care and Use of Experimental Animals.

All surgical and euthanasia procedures were carried out under ketamine at 90 mg/kg IM (Ketamidor; Richter Pharma GesmbH & CoKG, Wels, Austria) and xylazine at 10 mg/kg IM (Bayer AG, Leverkusen, Germany) anesthesia. The body temperature was monitored and maintained at 37 °C during anesthesia. The hypotension procedure was performed on day 15 of gestation as described previously (22). Briefly, the right femoral artery was cannulated and attached to a pressure transducer (BIOPAC SS13L, BIOPAC Systems Inc., CA, USA). Following 30 min of stabilization, systemic hypotension was created by blood withdrawal (0.8–1 mL/100 g body weight) within 5 min to lower the initial mean arterial blood pressures (MABP) (80–85 mmHg for the control groups, 84–90 mmHg for the hypotension groups) to approximately 50 mmHg. The MABP was continuously monitored and maintained at the aimed value of 50 mmHg for a period of 30 min by withdrawal and reinfusion of blood during the hypotension period. The MABP was raised to presurgery levels by reinfusion of the collected blood over 10 min after a 30-min-long hypotension and the procedure was terminated as the animal was stabilized. All the procedures were performed in the control animals except for the blood withdrawal and creation of hypotension. Animals in the control and hypotension groups were transferred to their cages and allowed to recover. Both the control and hypotensive animals were allocated into 4 groups based on time of euthanasia after the procedure (0, 6, 12, and 24 h; n = 5 in each group). All animals were euthanized following cesarean section at the corresponding times for their experimental groups. Two randomly chosen fetuses (N = 10 in each group) from each mother were decapitated and processed for morphological studies. n/N represent mother/ fetus numbers in the experimental groups, respectively.

2.2. Histopathology and immunohistochemistry

Brain specimens, after fixation in 10% buffered formaldehyde (Merck, Darmstadt, Germany), were processed according to the routine tissue processing technique and embedded in paraffin. Next 5-μm serial coronal brain sections were examined and photographed using a Leica 6000 microscope analyzing system. All the coronal brain sections were evaluated at 100× and 400× magnification by 2 examiners blinded for the groups. The presence of edema, hemorrhage, or necrosis was sought and semiquantitatively scored as absent, + (mild), ++ (moderate), or +++ (severe). For the evaluation of apoptosis, the TUNEL (Terminal Transferase Mediated–dUTP Nick End Labeling) method (ApopTag Kit, Appligene Oncor, France) and a caspase-3 immunohistochemistry kit (23,24) (cleaved caspase-3 antibody 1:100; Oncogene, San Diego, USA and Zymed Histostain kit) were applied to consecutive serial sections. TUNEL (+) and caspase 3 (+) cells detected on the walls of the ventricles were counted in 10 serial sections from each brain specimen at 400× magnification twice by 2 separate examiners blinded for the groups. The mean of the counts of the 2 examiners was used for the final evaluation of the number of TUNEL (+) and caspase 3 (+) cells.

2.3. Statistical Analysis

SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. A normal distribution of the data was confirmed using the Kolmogorov–Smirnov test. Data are presented as mean ± SEM. Group comparisons of initial animal characteristics were performed with analysis of variance (ANOVA) followed by a post-hoc Tukey test. Pre- and posthypotension MABP values were compared using a paired t-test. Intragroup comparisons of histopathological data were evaluated with ANOVA followed by a post-hoc Tukey test. Intergroup comparisons of histopathological data between the control and hypotension groups were compared using Student’s t-test for independent samples. Differences were considered significant at P < 0.05.

3. Results

3.1. Experimental animals

The body weights of all the pregnant rats and the number of fetuses were comparable among the groups. The hypotension and control groups were similar in terms of initial MABP (Table 1). Systemic hypotension was successfully induced in the hypotension group as indicated by significantly lower MABP levels during the hypotensive period compared to the initial values (Table 1).
3.2. Histopathological and immunohistochemical evaluation of brain sections
The tissue architecture and structure in the control and hypotension groups was consistent with developmental stage in all brain specimens taken at specified time points. Accordingly, the neuroepithelium and cells migrating from the neuroepithelium were observed in the walls of the ventricles in both groups, and brain regions could not be completely distinguished on day 15. In addition, there was no marked difference between the hypotension and control groups in terms of normal development. Microscopic evaluation of brain specimens revealed only mild edema in some samples of the hypotension group at 12 h and 24 h. The edema was reduced at 24 h compared to 12 h.

3.3. Evaluation of apoptosis in brain sections
TUNEL (+) and caspase (+) cells were detected only on the walls of the ventricles at all time points for the control and hypotension groups (Tables 2 and 3).

TUNEL (+) cells were more abundant in all (0 h, 6 h, 12 h, and 24 h) hypotension groups when compared to the corresponding control groups (P < 0.05) (Figure 1; Table 2). In the hypotension groups, despite the detection of TUNEL (+) cells as early as 0 h and 6 h posthypotension, the number of TUNEL (+) cells were more numerous at 12 h when compared with the other time points in both the hypotension and the control groups (P < 0.05), and significantly declined at 24 h (Table 2).

Caspase-3 (+) cells were more prominent in the hypotension groups at all time points compared to the control groups (P < 0.05) (Table 3).

Table 1. Body weights, fetus numbers, and MABP levels of all groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Hypotension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>243 ± 3</td>
<td>288 ± 8</td>
</tr>
<tr>
<td>0 h</td>
<td>249 ± 6</td>
<td>272 ± 21</td>
</tr>
<tr>
<td>6 h</td>
<td>242 ± 7</td>
<td>250 ± 12</td>
</tr>
<tr>
<td>12 h</td>
<td>240 ± 4</td>
<td>278 ± 12</td>
</tr>
<tr>
<td>24 h</td>
<td>272 ± 21</td>
<td></td>
</tr>
<tr>
<td>Number of fetuses</td>
<td>9 ± 0.8</td>
<td>10 ± 0.7</td>
</tr>
<tr>
<td>0 h</td>
<td>11 ± 0.6</td>
<td>8.7 ± 1.6</td>
</tr>
<tr>
<td>6 h</td>
<td>9.8 ± 0.9</td>
<td>9.7 ± 0.9</td>
</tr>
<tr>
<td>12 h</td>
<td>7.8 ± 1.6</td>
<td>10 ± 0.7</td>
</tr>
<tr>
<td>24 h</td>
<td>7.8 ± 0.9</td>
<td>9.6 ± 0.9</td>
</tr>
<tr>
<td>Initial MABP (mmHg)</td>
<td>82.8 ± 2.5</td>
<td>80.1 ± 3.8</td>
</tr>
<tr>
<td>0 h</td>
<td>83.4 ± 3.9</td>
<td>88.1 ± 3.0</td>
</tr>
<tr>
<td>6 h</td>
<td>84.5 ± 3.7</td>
<td>83.7 ± 5.1</td>
</tr>
<tr>
<td>12 h</td>
<td>79.5 ± 3.9</td>
<td>87.0 ± 6.2</td>
</tr>
<tr>
<td>24 h</td>
<td>90.1 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>MABP during hypotension (mmHg)</td>
<td>54.5 ± 1.4'</td>
<td>53.6 ± 2.3'</td>
</tr>
<tr>
<td>0 h</td>
<td>53.6 ± 2.3'</td>
<td>50.6 ± 1.9'</td>
</tr>
<tr>
<td>6 h</td>
<td>54.6 ± 2.7'</td>
<td></td>
</tr>
<tr>
<td>12 h</td>
<td>50.6 ± 1.9'</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>54.6 ± 2.7'</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. (n = 5 pregnant animals/group). MABP = mean arterial blood pressure, *= significant difference vs. corresponding initial MABP.

Table 2. Numbers of TUNEL (+) cells in rat brains.

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.6 ± 0.3</td>
<td>0.6 ± 0.2</td>
<td>6.7 ± 0.9''</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Hypotension</td>
<td>3.0 ± 0.4'</td>
<td>2.4 ± 0.4'</td>
<td>12.2 ± 2.2''</td>
<td>5.8 ± 0.9''</td>
</tr>
</tbody>
</table>

Data are given as mean ± SEM (N = 10 fetuses/group). P < 0.05 * vs. corresponding controls, '' vs. 0 h, 6 h, and 24 h groups.

Table 3. Numbers of active caspase-3 (+) cells in rat brains.

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>2.2 ± 0.7''</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Hypotension</td>
<td>0.6 ± 0.2'</td>
<td>0.8 ± 0.3'</td>
<td>84.2 ± 36.8''</td>
<td>5.6 ± 2.2'</td>
</tr>
</tbody>
</table>

Data are given as mean ± SEM (N = 10 fetuses/group). P < 0.05 * vs. corresponding controls, '' vs. 0 h, 6 h, and 24 h groups, and P = 0.05 ' vs. corresponding control.
corresponding control groups (P < 0.05) (Figure 2; Table 3). Both control and hypotension groups showed a few caspase 3 (+) neurons at 0 h and 6 h. The most prominent increase in the number of caspase-3 (+) cells was observed at 12 h when compared with the other time points, both in the hypotension and the control groups (P < 0.05). The numbers of caspase-3 (+) cells were significantly lower at 24 h in both the hypotension and the control groups compared to at 12 h (Table 3).

Figure 1. TUNEL (+) apoptotic cells (black arrows) are most prominent in the hypotension groups (B = 6 h, D = 12 h, and F = 24 h after the procedure) when compared with the control groups (A = 6 h, C = 12 h, and E = 24 h after the procedure) (TUNEL-methyl green × 400).

Figure 2. Caspase-3 (+) cells (black arrows) are most prominent in the hypotension groups (B = 6 h, D = 12 h, and F = 24 h after the procedure) when compared with the control groups (A = 6 h, C = 12 h, and E = 24 h after the procedure). Many caspase-3 immunoreactive cells are seen in the 12-h hypotension group (Caspase-3, Hematoxylin ×400).
4. Discussion
Hypoxic ischemic brain damage commonly evolves over a period of time after an initial insult rather than occurring immediately. This gives an opportunity for therapeutic measures (10,25). A number of studies using different models have demonstrated the temporal pattern of neuronal death after HI. The results of this study represent the effect of HI experienced on day 15 of gestation, as in certain previous reports (7,9). An HI episode appears to trigger the apoptotic pathway, as has been shown in many experimental and human studies (17,19,20,26). The process of cell death depends on the strain, age, and developmental stage of the host (10,17,19). In neonatal HI, caspase-3 activation begins between 12 h and 24 h and continues for 7 days (19,20). A neonatal ischemia–reperfusion model of transient unilateral cerebral ischemia with permanent and/or transient occlusion of artery(ies) in 7-day-old rats showed the persistence of TUNEL (+) nuclei from 4 h to 30 days after reperfusion. Although the number of apoptotic cells remained stable until 96 h, they progressively decreased from 7 to 30 days indicating that cell damage in this model is a persistent and ongoing process (27). Romanko et al. detected calpain activity as early as 4 h and up to 48 h after reperfusion, while caspase-3 activation did not occur until 8 h and peaked at 24 h after HI (28). In a HI model similar to the current study, TUNEL (+) apoptotic cells were detected to persist at both 1 and 28 days postnatal (9). Since the ongoing discussion has not focused on the early intrauterine period but basically on postnatal days, we intended to investigate the time course of apoptotic cell death starting within the first 24 h following intrauterine hypotension. Different studies have conflicting results not only for the detection time of apoptosis but also the area affected. A postnatal combined left common carotid artery ligation with systemic hypoxia in rats (varying from 7 to 60 days postnatal) resulted in regional and temporal variations. For example, in the frontal lobe, retrosplenial cortex, caudate putamen, and globus pallidus, the density of apoptotic cells increased 12 h after HI, declined at 24 h and remained high for 7 days, whereas in the cingulate cortex, CA3, dentate gyrus, subiculum, laterodorsal thalamus, and medial habenula, the density of apoptotic cells peaked between 24 and 72 h after HI and then declined (19). Russell et al. exhibited increased caspase-3 activity at 12 h post-HI in the ipsilateral cerebral cortex and at 24 h in the hippocampus (CA1 region) (20). Furthermore, the size of the area of the neuronal death may vary depending on the duration and severity of the HI (17). We have chosen a transient systemic maternal hypotension model to create HI, since this is a common experience during the natural course of pregnancy mainly due to altered baroreceptor sensitivity (29), and aimed to examine the early effects of HI during the intrauterine period. It has been suggested by Vexler and Ferriero that major responses to injury and cell death mechanisms are different in the neonatal brain, favoring more apoptotic features (12). The current results indicate caspase-dependent apoptosis without ruling out a possible contribution of caspase-independent pathways. Our observations indicate a higher number of TUNEL (+) and caspase (+) cells at 12 h compared with the other time points both in the control and the hypotension groups. It is known that more than half of the initially formed neurons are deleted during normal development (30); therefore, it is expected that more apoptotic cells would be detected at some periods of differentiation and development. In support of our findings, it was suggested that the extent and properties of apoptosis of developing neurons may vary greatly at different stages of development and among different neuronal populations (30). It was also shown that prenatal stress was associated with an increased activity of caspase-3 during early postnatal development (31) and caspase-3-like activity in control animals exhibited a significant and rapid decline between postnatal day 10 and day 13 (26). Although our control animals were not exposed to hypotension, the anesthesia and sham operation could have accelerated the apoptotic process of normal neural development, but since all the animals experienced the same experimental conditions, the impact of HI is clear in the hypotension groups with statistical significance at the different time points.

Since the accumulated data indicate that the consequences of various insults depend on regional distribution, and the susceptibility of different brain regions varies according to developmental stage, when we tried to evaluate our results for regional distribution of apoptotic cells, an accurate mapping could not be performed due to the developmental stage of the fetal brain (32). Our results demonstrated apoptosis around lateral ventricular walls as early as 0 and 6 h after hypotension, reaching the highest degree at 12 h, and declining between 12 and 24 h. In accordance with our results, the subventricular zone (SVZ) is reported among the regions vulnerable to prenatal injury. In the study by Romanko et al., activated calpains and caspase-3 colocalized in the lateral SVZ, an area rich in progenitor cells, but not in the medial SVZ; this type of injury may alter brain development and create long-term and more widespread effects (28,33). The activation of caspase-3 may be more extensive in HI-induced neuronal death in immature neurons compared to mature neurons (17,19) and susceptibility of neurons to injuries varies with age (17). In line with these reports, our results suggest that activation of caspase-3 greatly contributes to the HI-induced death of immature neurons by apoptosis, which is especially prominent at 12 h after the insult during the intrauterine period. Our findings also emphasize the
influence of the stage of intrauterine brain development on the time course and degree of brain injury. This is an area that needs to be studied further to determine the susceptibility of the developing brain to HI at a certain time since the immature brain responds differently to treatment than does the mature brain (34).

The results of the present study suggest that prenatal HI exposure derived from systemic maternal hypotension causes increased apoptotic neuronal cell death in rat pups. In addition, our findings may help to determine the therapeutic time window for neuroprotective drugs in treating HI brain injury. The impact becomes visible very early after the injury, and so any possible intervention should be considered for use during the prenatal period in addition to the widespread postnatal application, and the therapeutic window should be evaluated separately for each developmental stage in order to develop effective measures for treatment or neuroprotection.

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References


