Indomethacin Prevents Neuronal Apoptosis in Newborn Rats with Hypoxic-Ischemic Brain Injury

İndometazin Hipoksik-İskemik Beyin Hasarlı Yenidoğan Ratlardaki Nöronal Apopitozisi Önler

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ABSTRACT Objective: Cyclooxygenase pathway and prostaglandins play an important role in the pathogenesis and delayed mechanisms of hypoxic-ischemic brain injury. The aim of this study was to investigate the effect of different doses of indomethacin, a nonselective cyclooxygenase inhibitor, on neuronal apoptosis in rats with hypoxic-ischemic brain injury. **Material and Methods:** Seven-day-old rat pups with the Rice model of hypoxic-ischemic cerebral injury were randomly divided into five groups. Group 1 (n= 15) pups were given physiologic saline, neither ligation nor hypoxia were performed. Group 2 (n= 15) pups were treated with physiologic saline after hypoxic-ischemia. Group 3 (n= 15) pups were treated with indomethacin at a dose of 2 mg/kg before hypoxic ischemia. Group 4 (n= 15) pups were treated with three doses of indomethacin at a dose of 2 mg/kg every 12 h after hypoxic-ischemia. Group 5 (n= 15) pups were treated with three doses of indomethacin, at a dose of 4mg/kg every 12 h after hypoxic ischemia. After 72 hours, the rats were decapitated and brain hemispheres were evaluated by the TUNEL (Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) staining method. **Results:** Indomethacin treatment, either before or after hypoxia, resulted in a significant reduction in the numbers of apoptotic cells in the rat brain when compared to those who were treated with physiologic saline after hypoxic-ischemia (P< 0.001). **Conclusion:** Our results demonstrated that indomethacin administration, either before or after hypoxic-ischemia, reduces neuronal apoptosis; and we propose that indomethacin may be a potential choice of treatment for hypoxic-ischemic brain injury.

Key Words: Hypoxia-ischemia, brain; apoptosis; indomethacin

ÖZET Amaç: Siklooksijenaz yolağı ve prostaglandinler, hipoksik-iskemik beyin hasarının patogenezinde ve geç dönemki sürecinde önemli rol oynamaktadır. Bu çalışmanın amacı, bir non-selektif siklooksijenaz inhibitörü olan indometazini farklı dozlarda uygularak hipoksik-iskemik beyin hasarlı ratlarda nöronal apopitozis üzerine etkilerini araştırmaktır. **Gereç ve Yöntemler:** Levine-Rice metoduna göre hipoksik iskemi oluşturulan yedi günlük rat yavruları rastgele beş gruba ayrıldı. Grup 1'deki (n: 15) yavrulara ne ligasyon ne de hipoksi uygulanmadan sadece serum fizyolojik verildi. Grup 2'deki (n: 15) yavrulara hipoksik-iskemik hale getirildikten sonra serum fizyolojik verildi. Grup 3'teki (n: 15) yavrulara hipoksik-iskemik hale getirilmeden önce 2 mg/kg tek doz indometazin verildi. Grup 4' teki (n: 15) yavrulara hipoksik-iskemik hale getirildikten sonra 12 saatte bir 2 mg/kg dan toplam üç doz indometazin verildi. Grup 5'teki (n: 15) yavrulara hipoksik-iskemik hale getirildikten sonra 12 satte bir 4 mg/kg dan toplam üç doz indometazin verildi. Ratların 72 saat sonra kafaları ayrılarak beyin hemisferlerine ait dokular TUNEL (Terminal deoksinükleotidil transferaz aracılı dUTP nick sonu etiketleme) boyama metodu ile değerlendirildi. **Bulgular:** Hipoksi öncesi veya sonrasındaki indometazin tedavisi alan rat yavrularında, hipoksik iskemi sonrası serum fizyolojik verilen ratlara göre beyindeki apopitotik hücre sayısında anlamlı derecede azalma ile sonuçlanmıştır (P< 0.001). **Sonuç:** Çalışmamızın bulgularına gore, hipoksik iskemi öncesi veya sonrasında verilen indometazin nöronal apopitozisi azaltmakta olup bu bağlamda hipoksik-iskemik beyin hasarı tedavisinde indometazinin potansiyel bir tedavi seçimi olabileceğini düşünmekteyiz.

Anahtar Kelimeler: Hipoksi- iskemi, beyin; apoptoz; indometazin

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Hypoxic ischemic brain injury remains one of the most important neurologic complications in the newborn.[1] Despite the fact that hypoxic-ischemic brain injury closely corresponds to experimental models of cerebral hypoxia-ischemia where successful neuroprotective interventions were introduced, currently no agent has been proven valuable to improve the chronic sequelae of hypoxic-ischemic brain injury in the clinical setting.[2,3]

It is well-known that the cyclooxygenase pathway becomes the major source of free radicals following exposure to certain types of oxidative stress, such as ischemic insult to the brain.[4] Recent studies have shown that the presence of reactive oxygen species is closely associated with DNA fragmentation.[5] In addition, the process of delayed neuronal death after brain ischemia is often reported as apoptosis because of the associated DNA fragmentation.[6,7]

Indomethacin, a cyclooxygenase inhibitor of prostaglandin (PG) synthesis, has been suggested to have a protective effect on hypoxic-ischemic brain injury in several animal studies.[9-11] However, there is no consensus on the dose of indomethacin. In this respect, we aimed to investigate the effect of different doses of indomethacin on apoptosis by TUNEL method in the newborn rat brain with hypoxic-ischemic (H-I) injury.

MATERIAL AND METHODS

Seven-day-old Wistar albino rats of either sex were used in the study. A total of 60 animals were obtained from Cukurova University, Institute of Medical Experiments Research and Practice Laboratory, and were randomly divided into five groups by using a computer-generated random number table. The study was approved by the Ethics Committee of the Medical and Experimental Research Center of the Medical Faculty.

EXPERIMENTAL PROCEDURE

Rats were anesthetized by halothane inhalation (3.0% for induction and 1.0-1.5% for maintenance). A median incision was made in the neck. The left carotid artery (ipsilateral) was identified after careful dissection and was ligated with a 4/0 silk suture. After the wound was sutured, the animals were allowed a recovery period of 2.5 h. Rats in all groups except the sham group were then placed in a plastic chamber and exposed to a continuous flow of 8% oxygen and 92% nitrogen for 2 h to induce systemic hypoxia according to the method used by Rice et al.[12] After H-I insult, the rats were returned to their dams following a 30 min reoxygenation period and kept in a room (12:12 h light/dark cycle) until decapitation at the age of 10 days. After these procedures, under deep intraperitoneal thiopental sodium (50 mg/kg) anesthesia, all the pups were decapitated at the age of 10 days and the extracted brain tissues were removed for pathological evaluation.

Group 1: Sham group (n= 15): After a median neck incision was made, the carotid artery was identified and dissected, but neither ligation nor hypoxia was performed. Animals were given intraperitoneal physiologic saline at a dose of 100 µl per 12, total three doses, 30 minutes after neck incision.

Group 2: Hypoxic-ischemic group (n= 15, served as H-I): Rats were given intraperitoneal physiologic saline at a dose of 100 µl per 12, total three doses, 30 minutes after hypoxic ischemia.

Group 3: Indomethacin administered at a dose of 2 mg/kg before H-I group (n= 15, served as IND 2 mg before H-I): The rats were administered 50 mg intraperitoneal indomethacin (Confortid, Alpharma-ISIS, Germany) within a distillated water solution at a single dose of 2 mg/kg (a dose of 100 µl) 30 minutes before hypoxic ischemia.

Group 4: Indomethacin administered at a dose of 2 mg/kg after H-I group (n= 15, served as IND 2 mg after H-I): The rats were administered 50 mg intraperitoneal indomethacin within a distillated water solution at a dose of 2 mg/kg (a dose of 100 µl) per 12 h, total three doses, 30 minutes after hypoxic ischemia.

Group 5: Indomethacin administered at a dose of 4 mg/kg after H-I group (n= 15, served as IND 4 mg after H-I): The rats were administered 50 mg intraperitoneal indomethacin within a distillated

water solution at a dose of 4 mg/kg (a dose of 100 µl) per 12 h, total three doses, 30 minutes after hypoxic ischemia.

TUNEL (TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE-MEDIATED dUTP NICK END LABELING) METHOD

Apoptotic cell death was detected using an in situ Apoptosis Detection Kit (Chemicon, USA) according to the manufacturer's instructions. Briefly, sections were deparaffinized and rehydrated and pretreated with proteinase K for 15 min at room temperature, then endogen peroxidase activity was quenched with 3% H_2O_2. Slices were then incubated at 37°C for 60 min in a moist chamber with 50 µl of TdT buffer. The reaction was visualized by Streptavidin HRP solution and detected with 0.05% DAB. TUNEL labeled slides were counterstained with 1% methyl green.

PATHOLOGICAL EVALUATION

Rat brains were sliced with an example at the level of the hippocampus. This zone was prepared from the paraffin blocks serial sections that were previously made. Sections to be processed with S7100 ApopTag® peroxidase were evaluated. An experienced pathologist counted apoptotic cells in the region of the hippocampus CA1 and parietal cortex of both right and left hemispheres. TUNEL positive stained cells were calculated in five power fields (10 X 40) under the light microscope.

STATISTICAL ANALYSIS

Descriptive statistics (mean and standard deviation) were used to estimate all parameters in each group. A Tukey's test was used for multiple comparisons in one-way ANOVAs because it showed normal distrubution, and a Games-Howell test was used for multiple comparisons in Welch test statistics. The results were considered statistically significant at $P< 0.05$.

RESULTS

Table and figures denote TUNEL-positive apoptotic cells of each group (Table 1, Figures 1, 2). At 72 hours of hypoxic ischemia (H-I), the TUNEL-positive cell number was 22.00 ± 5.28, while it was 3.86 ± 1.09 in the sham group ($P< 0.001$). Apoptotic cell number was reduced by nearly half with 2 mg/kg indomethacin treatment both before ($P< 0.001$) and after ($P< 0.001$) H-I when compared to the H-I group. TUNEL-positive cell number, in the 4 mg/kg indomethacin after H-I treatment group declined to a similar level with the apoptotic cell number in the sham group ($P< 0.05$). Among the treatment

TABLE 1: Count of TUNEL-positive stained cells in study groups.

Groups	Count of TUNEL-positive stained cells (Mean ± SD)		
	Contralateral hemisphere	Ipsilateral hemisphere	p
1 (Sham) (n= 15)	3.90 ± 1.10 (2-5)	3.53 ± 1.06 (2-5)	>0.05
2 (Hypoxic-ischemic) (n= 15)	12.40 ± 1.58 (11-14)	31.46 ± 10.97 (13-47)	<0.01*
3 (Indomethacin 2 mg/kg before H-I) (n= 15)	8.26 ± 1.03 (6-10)	11.33 ± 1.58 (10-14)	<0.05*
4 (Indomethacin 2 mg/kg after H-I) (n= 15)	7.93 ± 0.79 (6-10)	11.00 ± 0.65 (10-12)	<0.05*
5 (Indomethacin 4 mg/kg after H-I) (n= 15)	5.86 ± 0.91 (5-7)	6.60 ± 1.40 (5-8)	>0.05

Indomethacin treatment either before or after hypoxia resulted in statistically significant reduction of the numbers of apoptotic cells in both hemispheres, when compared to H-I group ($P< 0.05$).

*There were statistically significant differences in the H-I groups, IND 2mg before H-I, and IND 2mg after H-I TUNEL positive cells counts between the contralateral hemisphere and ipsilateral hemisphere ($P< 0.01$, $P< 0.05$, and $P< 0.05$ respectively).

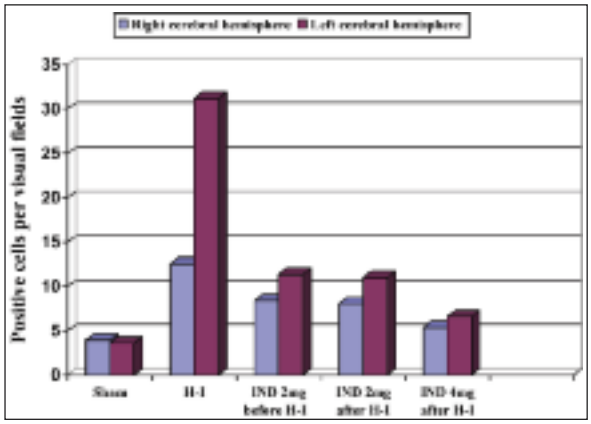

FIGURE 1: TUNEL-positive cells in study groups.

groups, the best neuroprotective effect was achieved with 4 mg/kg indomethacin treatment after H-I. A single dose of 2 mg/kg indomethacin treatment before H-I actualized a quite successful neuroprotective effect similar to treatment with 2 mg/kg indomethacin after H-I.

As far as the effects of indomethacin on hypoxia and H-I in brain hemispheres were concerned, we found that apoptotic cell number was significantly higher in the ipsilateral hemisphere than in the contralateral hemisphere in all groups except the sham and post H-I 4 mg/kg indomethacin treatment groups (P< 0.05). In the ipsilateral hemisphere, the apoptotic cell number was reduced three-fold with 2 mg/kg indomethacin treatments before and after H-I (P< 0.01), whereas the apoptotic cell number was reduced nearly five-fold with 4 mg/kg indomethacin treatment after H-I when compared to the H-I group (P< 0.001).

DISCUSSION

Neonatal hypoxic-ischemic brain injury induced by birth asphyxia is one of the most frequent perinatal problems and sometimes leads to severe neurological sequelae.[13,14] Neuronal injury after neonatal hypoxic-ischemic brain damage consists of necrosis and especially apoptosis.[15] To date, there have not been any treatments proven to be effective, and only supportive therapy has been available.

During hypoxic ischemia, arachidonic acid and prostaglandin metabolism play an important role in neuronal apoptosis. Upregulation of neuronal cyclooxygenase-2 (COX-2) and the elevation of

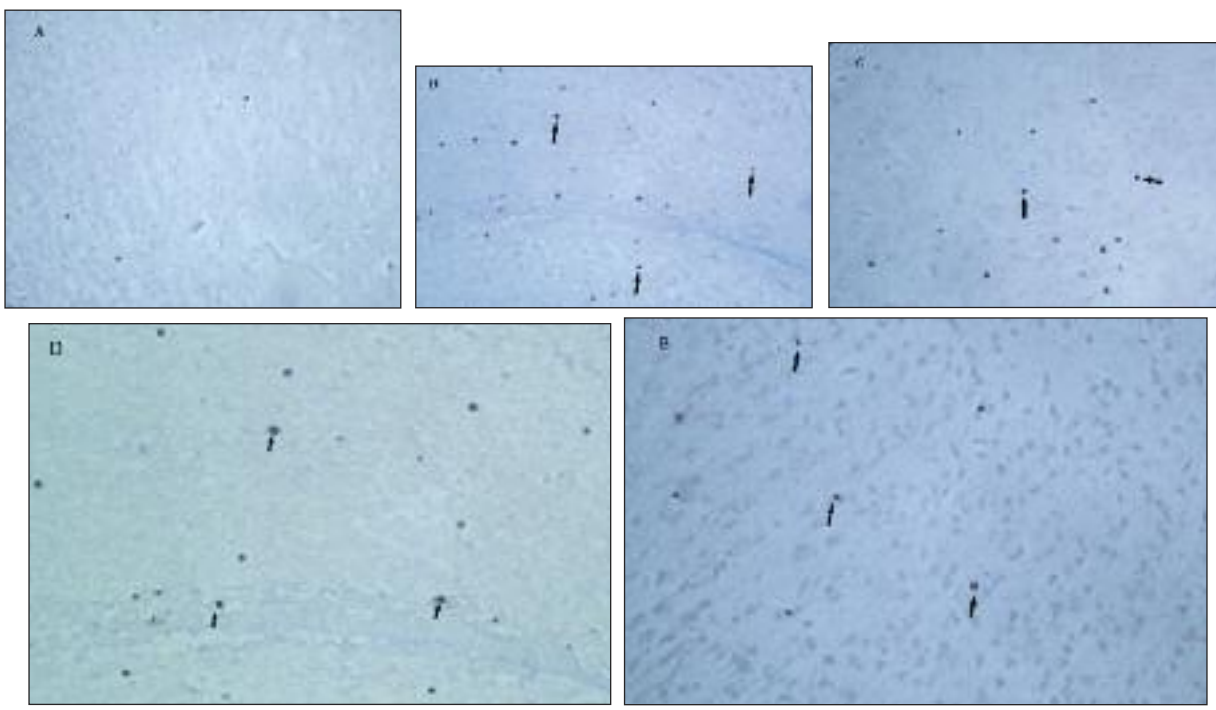

FIGURE 2: TUNEL-positive apoptotic cells in hippocampus in the **(A)** sham group, **(B)** hypoxic ischemic group, **(C)** IND 2 mg before H-I group, **(D)** IND 2 mg after H-I group, **(E)** IND 4 mg after H-I group. Original magnification, x400.

prostaglandin E_2 (PGE_2) have been reported to occur after cerebral ischemic insult.[16,17] Iadecola et al.[18] have demonstrated reduced and increased susceptibility to ischemic brain injury in COX-2 and COX-1-deficient mice. In addition, several studies have suggested that PGE_2 induces caspase-dependent apoptosis, acting via an EP2-like receptor, and a signaling mediator involved in hippocampal excitatory glutaminergic synaptic transmission in rat cultured cortical neurons.[19,20]

It has been demonstrated that indomethacin, a cyclooxygenase inhibitor of prostaglandin synthesis, inhibits delayed neuronal damage and decreases brain infarct volume, especially DNA damage. In the same way, indomethacin prevents delayed neuronal death.[10,11] Buccelatti et al.[11] demonstrated that indomethacin treatment with 20 mg/kg/dose one hour prior to ischemia decreased infarct size in focal ischemic rat brain compared to the control group. Tutak et al.[9] reported that treatment with 0.2 mg/kg indomethacin (total three doses every 12 hours) 30 minutes after H-I decreased infarct size in experimental hypoxic ischemic rat brain compared to the control group. In addition, Kondo et al.[10] showed that TUNEL-positive stained apoptotic cell number was reduced significantly with 5 mg/kg indomethacin administered five minutes before ischemia when compared to control group, and thus delayed neural damage was prevented in a gerbil model of transient forebrain ischemia. In the present study, we found that apoptotic cell number in an H-I rat model was significantly lower than the control group with a single dose of 2 mg/kg indomethacin therapy 30 minutes prior to H-I. Additionally, 2 mg/kg indomethacin administration three times with 12-hour intervals after H-I was found to reduce apoptotic cell number and it produced the same neuroprotective effect as observed with 2 mg/kg indomethacin pre H-I treatment. Single-dose indomethacin administration after H-I was not performed in our study. Thus, we could not establish the consequences of the effect of post H-I single dose indomethacin on rat brain.

Our study concluded that treatment with a dose of 4 mg/kg indomethacin 30 minutes after H-I prevented apoptosis more effectively than treatment with 2 mg/kg/dose indomethacin before and after H-I. Surprisingly, treatment with high-dose indomethacin after H-I reduced the degree of delayed neuronal damage to the levels of hypoxia-induced neuronal damage. Previous studies suggested that there was a relationship between PGE_2 levels and delayed neuronal damage, and PGE-induced neuronal apoptosis was believed to occur in a dose dependent manner.[19] Our study suggested that the dose-dependent neuroprotective effects of high-dose indomethacin therapy might be associated with increased cyclooxygenase inhibition due to decreased prostaglandin products and free radicals. Further studies on this issue are required, in our opinion.

In conclusion, we demonstrated that both pre-ischemic and post-ischemic administration of indomethacin prevented delayed neuronal death. In particular, it was shown that high-dose indomethacin at a 4 mg/kg dose had a more neuroprotective effect than low-dose indomethacin. However, further studies are needed to fully address the neuroprotective effect of indomethacin on apoptosis in hypoxic-ischemic encephalopathy and optimize the treatment protocol to maximize its therapeutic activity.

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