Protective Effects of Platelet-Activating Factor Antagonist ABT-491 on the Peripheral Nerves in Hypoxic Ischemia-Induced Neonatal Rat Model

Hipoksik İskemiye Maruz Kalan Yenidoğan Sıçanlarda, Platelet Aktive Edici Faktör Antagonisti Olan ABT-491'in Periferik Sinirler Üzerinde Koruyucu Etkisi

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ABSTRACT Objective: Neonatal hypoxic-ischemic (HI) insult has acute and long term deleterious effects on many tissues including the peripheral nerves. To date, no study has investigated the role of platelet-activating factor (PAF) antagonists on peripheral nerve damage in a neonatal rat model of HI. In this study, we examined the effects of PAF antagonist (ABT-491) on peripheral nerve damage in rats in the 16th week that were exposed to HI on 7th day after birth. **Material and Methods:** Seven-day-old Wistar rat pups were subjected to right common carotid artery ligation and hypoxia (92% nitrogen and 8% oxygen) for one hour. They were treated either with ABT-491 (n=19) or saline (n=20) immediately after hypoxia. In sham group (n=20), neither ligation nor hypoxia was performed. The compound motor action potential (CMAP) recordings of all animals were made in the sixteenth week following the HI. For CMAP recordings, bipolar stimulating electrodes were placed on the sciatic nerve. Upon stimulation, two surface electrodes, placed over the gastrocnemius muscle, recorded compound muscle action potentials. **Results:** The amplitude of CMAP recorded from the rats treated with saline after hypoxia was smaller compared to the sham group. However, the CMAP recorded from the group treated with ABT-491 after HI was not significantly different from the sham group. **Conclusion:** This study implies that HI has axonal damage on peripheral nerve but a PAF antagonist ABT-491 has a preventive effect on the axonal dysfunction after HI.

Key Words : ABT-491; Action potentials; peripheral nerves; ischemia; anoxia

ÖZET Amaç: Neonatal hipoksik-iskemik (Hİ) hasar periferik sinirler dahil birçok dokuda kısa ve uzun vadede zararlı etkiler gösterir. Bugüne kadar, Hİ 'ye maruz kalan yenidoğan sıçanların periferik sinir hasarı üzerine platelet aktive edici faktör (PAF) antagonistinin rolü araştırılmamıştır. Bu çalışmada PAF antagonistinin (ABT-491) doğumdan sonraki yedinci günde Hİ'ye maruz kalan sıçanlarda 16. haftadaki periferik sinir hasarına etkilerini inceledik. **Gereç ve Yöntemler:** Yedi günlük Wistar sıçan yavruları sağ ortak karotid arter ligasyonuna ve bir saat süreyle hipoksiye (%92 nitrojen ve %8 oksijen) maruz bırakıldılar. Hipoksinin hemen ardından bir grup sıçana ABT-491 (ABT Grubu), diğer bir grup sıçana serum fizyolojik (Salin Grubu) verildi. Üçüncü grup sıçana (Sham Grubu) ligasyon veya hipoksi uygulanmadı. Bütün hayvanlarda Hİ'yi izleyen onaltıncı haftada bileşik motor aksiyon potansiyeli (CMAP) kayıtları yapıldı. Bu amaçla, bipolar uyarıcı elektrotlar siyatik sinir üzerine yerleştirildikten sonra uyarı verildi ve iki yüzeyel kaydedici elektrot ise gastrokinemus kası üzerine yerleştirilerek CMAP'lar kaydedildi. **Bulgular:** Hİ sonrası salin uygulanan sıçan grubundan kaydedilen CMAP amplitüdü Sham grubundan kaydedilen CMAP amplitüdlerine göre düşük bulundu. Buna karsılık, Hİ sonrası ABT-491 verilen grupta ölçülen amplitüd ile Sham grubundan ölçülen amplitüd arasında istatiksel olarak anlamlı fark bulunmadı. **Sonuç:** Bu çalışma periferik sinir üzerinde Hİ'nin axonal hasar oluşturduğunu, fakat bir PAF antagonisti olan ABT-491'in Hİ sonrasındaki aksonal bozukluk için koruyucu bir etki sağladığını göstermektedir.

Anahtar Kelimeler: ABT-491; Aksiyon potansiyelleri; periferik sinirler; iskemi; anoksi

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Hypoxic-ischemic brain injury (HIBI) that occurs during the perinatal period is one of the most commonly recognized causes of severe, long-term neurologic deficits in children.[1] Despite major improvements in perinatal medicine, the incidence of cerebral palsy attributed to intrapartum asphyxia has remained unchanged, because management strategies were supportive and not aimed to stop the ongoing injury. Recent studies focused on head or whole body cooling to decrease the severity of ongoing injury soon after perinatal hypoxic-ischemic (HI) insult and reported promising results.[2]

Perinatal and neonatal HI insult has acute and long term deleterious effects not only on brain but many other tissues including peripheral nerves. Although the mechanism of neuronal damage has not been exactly understood in neonatal HI, hypoxic ischemia systematically triggers a cascade of biochemical and molecular events including activation of neutrophils, membrane depolarization, increased intracellular calcium, production of lipid peroxidation and generation of oxygen-derived free radicals.[3] These events induce DNA damage, neuronal injury, neurodegeneration and cell death.[4] Preventing or treating brain and peripheral nerve injury together may be more effective in decreasing the complications of HI.

Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a phospholipid mediator that regulates the functions of a variety of cells not only in the peripheral tissues but also in the nervous system.[5] Several studies suggest that PAF might exaggerate pain.[6,7] PAF is produced by various kinds of cells including keratinocytes [8] and inflammatory cells,[9] and the level of PAF is increased in inflamed tissue,[10] In addition, during hypoxia, ischemia and in other pathologic conditions involving oxidative stress, PAF concentration increases and it becomes a pro-inflammatory messenger and a mediator of neurotoxicity.[11]

PAF-receptor antagonists elicit neuroprotection in various models of neural injury. Therefore, over the past decade, several investigations have been performed to investigate the role of PAF and PAF antagonists in hypoxic-ischemic brain injury.[12] However, no study has investigated the role of HI on peripheral nerves and the effects of PAF antagonists on peripheral neuronal damage in a neonatal rat model of HI.

The changes of cell membrane potential are detected as an action potential. Compound motor action potential (CMAP) is the summated activity of the synchronously activated muscle fibers in the muscle innervated by the axons and motor units represented in that muscle. Measurements of the peak-to-peak amplitude and the distal motor latency (DML) (thus, conduction velocity) of action potential may provide information about membrane sodium (Na) and potassium (K) transport. CMAP latency is the time required for the action potentials in the fastest conducting fibers to reach the nerve terminals and activate the muscle fibers. In addition, amplitude can be used to estimate the number of activated nerve fibrils. Therefore, CMAP provides a physiological assessment of the muscle fibers activated by the stimulus.[13]

The aim of this study was to evaluate whether HI had an influence on peripheral nerves in rats at four months of age that were exposed to hypoxic ischemia on 7th day after birth. Additionally, in this study, we investigated the efficacy of ABT-491, a highly potent and selective PAF antagonist, after HI. Hence, we evaluated the changes in action potential parameters of HI rats and compared them with ABT-491 administered ones immediately after HI insult.

MATERIAL AND METHODS

Seven-day-old Wistar male rat pups (n= 59), delivered spontaneously, were used in this experimental study. The brain of the rat at this stage is histologically similar to that of a 32-34-week gestation human fetus or human newborn infant at term, and this model has been proved to be useful in many studies.[14] All procedures were approved by the Medical Faculty Experimentation Ethics Committee on animal research at our institution and followed the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

ANIMAL PREPARATION AND SURGICAL PROCEDURE

The rats were randomly allotted into one of the three experimental groups: saline treated (ST) group, ABT-491 treated (ABT) group and sham (SH) group, ABT containing 19 animals and ST, SH containing 20 animals.

Rat pups in ST and ABT groups were anaesthetized by isoflurane inhalation and duration of anesthesia was less than 5 min. In these groups, HI was induced according to the Levine-Rice model.[15] A median incision was made in the neck. Under the microscopic magnification, the right common carotid artery was dissected and ligated with 6-zero silk sutures. After the wound was sutured, all the offspring rats were left for one hour next to the mother for the process of recovery and nutrition. Rats were then placed in a plastic chamber and exposed to a continuous flow of 8% oxygen-92% nitrogen for 1h. Saline (0.2 ml) was injected intraperitoneally immediately after hypoxia to the ST group. The ABT group rat pups were administered intraperitoneally 0.4 mg/kg ABT-491 which was dissolved in 0.2 ml of saline, immediately after hypoxia.

Then, the rats were allowed to have a 2 h recovery period in an open chamber without any supplemental oxygen. Rats in sham group were anesthetized by isoflurane inhalation. After a median neck incision was made, the right common carotid artery was found. However, neither ligation nor hypoxia was performed. The animals in the sham group were placed in an open chamber for the same intervals. The chambers were partially submerged in a water bath at 33 ± 1 °C to maintain a constant thermal environment.

ELECTROPHYSIOLOGICAL RECORDING

Body weights of all the animals were measured in the sixteenth week following the HI, just before electrophysiological recordings were made. Electrophysiological recordings (CMAP) across the nerve segment were made using BIOPAC MP 100 acquisition system (Santa Barbara, USA) (Figure 1). Prior to electrophysiological recordings, the rats were anesthetized with 80 mg/kg ketamine hydrochloride (Ketalar, Eczacibasi Ilac Sanayi ve Ticaret A.S., Istanbul, Turkey) and xylazine (8 mg/kg),

FIGURE 1: General EMG recording system.

administered intramuscularly. The hind limbs of the rats were placed in a standard position during the electrophysiological recordings. Then the hind limbs were shaved. CMAPs were recorded in all three groups. Standardized nerve conduction study techniques were used to record CMAP of the gastrocnemius muscle.[16] Briefly, bipolar stimulating electrodes (Medelec small bipolar nerve electrodes, 6894T, Oxford, UK) were placed around the sciatic nerve at sciatic notch. The supramaximal stimulus consisted of a single square pulse (intensity 10 V, duration 0.5 ms). The ground electrode was placed on the other thigh, to which the stimulation was not applied. The sciatic nerve was stimulated from the most distal site of stimulation by bipolar electrode (Figure 2). CMAPs were recorded from the gastrocnemius muscle by the surface disc electrodes (Medelec, number 017K006, Oxford, UK) which were always positioned on the distal one thirds of the leg. During the study, the body temperature of rats was maintained at 37 ^{0}C using a heating pad, and continuously monitored by rectal probe digital thermometer. BIOPAC Acknowledge Analysis Software (ACK 100 W) was used to measure CMAP peak-to-peak amplitude and distal motor latency. The peak-to-peak amplitude of a given CMAP was defined as the height in millivolts from the peak of the positive phase to the peak of the negative phase. The distal motor latency (in milliseconds) was determined as the interval of time between the onset of the stimulus and that of the response.

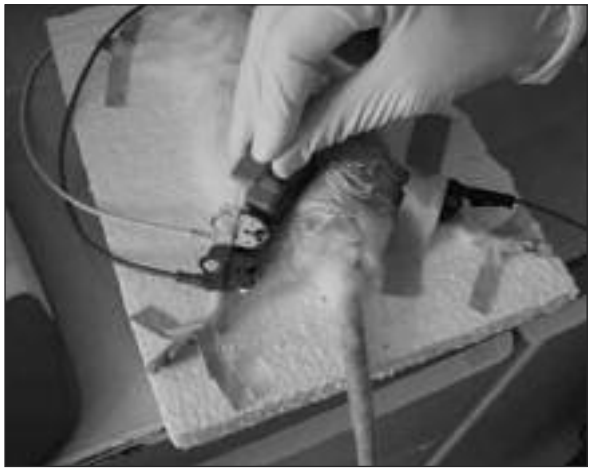

FIGURE 2: Arrangement for measuring distal motor latency in the sciatic nerve. The sciatic nerve was stimulated from the most distal site by bipolar electrode and the compound action potentials were recorded from the gastrocnemius muscle.

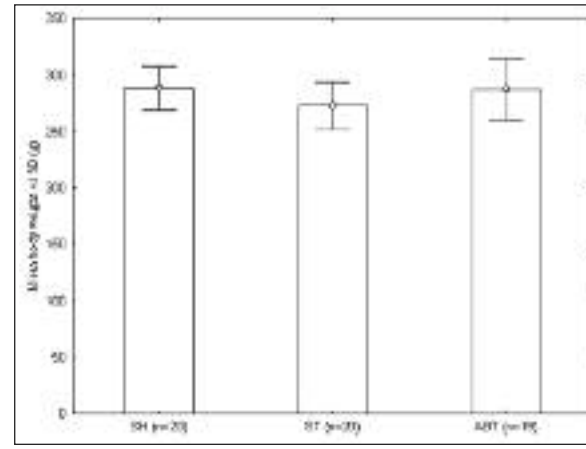

FIGURE 3: Body weight of the animals in the sixteenth week for sham (SH), saline treated (ST) and ABT-491 treated (ABT) group. Values are means ± standard deviation (SD). There was no statistically significant difference (p = 0.06) in the body weight values in all three groups in the sixteenth postnatal week.

After electrophysiological recording, all pups were euthanized by decapitation.

STATISTICAL ANALYSIS

The data were processed and analyzed using the statistical package STATISTICA 6.0 package. Descriptive data are presented as means and standard deviations (SD). The distribution of variables (body weight, peak-to-peak amplitude and distal motor latency) was investigated by using Shapiro Wilk's normality test and the comparisons were done using parametric tests. The differences between groups were tested using ANOVA and post-hoc Bonferroni tests. Significant differences (two-tailed p) less than 0.05 were regarded as significant.

RESULTS

BODY WEIGHT

The mean body weights of the rats in all three groups were not different at postnatal sixteenth week (Figure 3). The body weight values for rats in SH, ST and ABT groups were 288.48 ± 19.90 g; 272.80 ± 20.71 g and 287.23 ± 26.23 g, respectively.

ELECTROPHYSIOLOGICAL DATA

Peak-to-peak amplitude of compound motor action potential (CMAP amplitude): Typical records of CMAP in sham group, and saline-treated and ABT-491 treated hypoxic ischemic groups are shown in Figure 4. The means and standard deviations for CMAP amplitudes in all groups are given in Table 1 and illustrated in Figure 5. As seen in Table 1, Figure 4 and Figure 5, there were significant differences between the SH and ST groups regarding to CMAP amplitude.

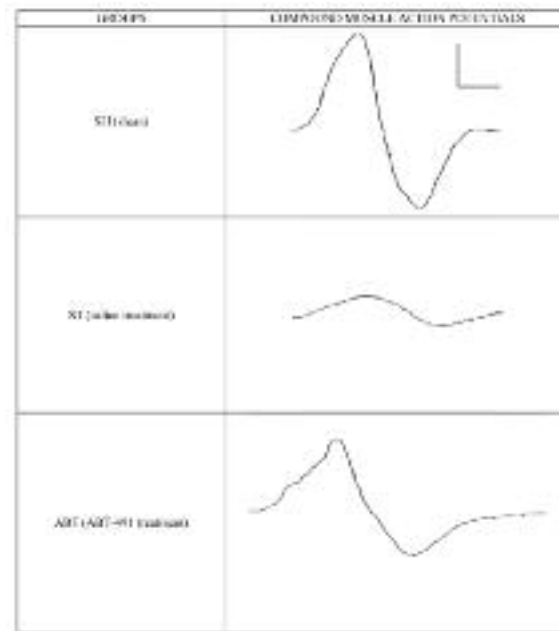

FIGURE 4: Sample records of the compound motor action potential (CMAP) in sham (SH), saline treated (ST) and ABT-491 treated (ABT) group. Calibrations for all traces are shown in the upper right; vertical bar = 3.75 mV; horizontal bar = 1.6 ms.

TABLE 1: Descriptive statistics for compound motor action potential parameters.

Variables	Experiments	Mean	SD
Peak-to-peak amplitude	Sham	15.57	2.19
(mV)	Saline treated	12.28**	3.02
	ABT-491 treated	13.94	2.81
Distal motor latency	Sham	1.30	0.14
(ms)	Saline treated	1.39	0.25
	ABT-491 treated	1.29	0.16

** p< 0.01 compared to the sham group. SD: Standart deviation.

In the sixteenth week following HI, EMG (electromyography) recordings in the ST group showed significantly lower values of CMAP in the gastrocnemius muscle when compared to normal values obtained in animals in the sham group (12.28 ± 3.02 mV compared to 15.57 ± 2.19 mV normal values, p< 0.01), suggesting a partially interruption of the signal through the nerve fibers and mainly reflecting axonal dysfunction (see Table 1, Figure 4 and Figure 5); but, as seen in Table 1, Figure 4 and Figure 5 there were no statistically significant differences between the sham and ABT treated groups (15.57 ± 2.19 mV and 13.94 ± 2.81 mV, respectively, p> 0.05) regarding to CMAP amplitude. ABT-491, applied just after HI prevents the decrease in the amplitude of CMAP in rats with HI (Figure 5) showing that CMAP amplitude was protected by the treatment with ABT-491.

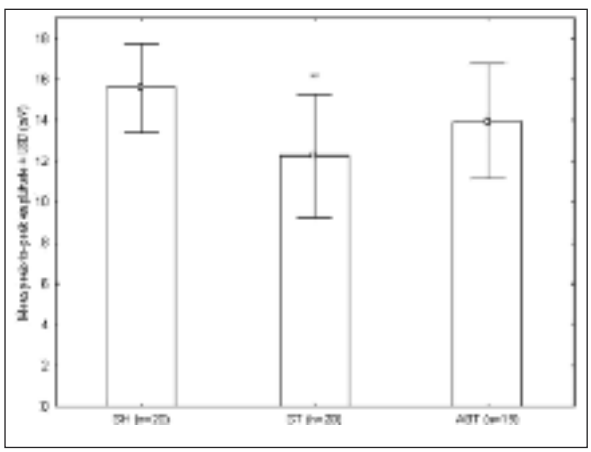

FIGURE 5: Peak-to-peak amplitude values with standard deviation (1SD) around the mean for sham (SH), saline treated (ST) and ABT-491 treated (ABT) groups. ** p < 0.01 compared to the sham group.

***Distal motor latency (DML)*:** Figure 6 shows the calculated means (with standard deviations) for DML of all groups. As seen in Table 1 and Figure 6, DML values did not show statistically significant differences (sham 1.30 ± 0.14 ms; ST 1.39 ± 0.25 ms; ABT 1.29 ± 0.16 ms; p> 0.05) among all groups.

DISCUSSION

In this study, ligation of the right common carotid artery and exposure to hypoxia by a continuous flow of 8% oxygen-92% nitrogen for 1h resulted in electrophysiological abnormalities in the peripheral nerve of the rats. This study demonstrates that HI results in a significant decrease in the peak-to-peak amplitude of CMAP in the gastrocnemius muscle, suggesting a partial interruption of the signal through the peripheral nerve fibers, mainly reflecting axonal dysfunction. However, a PAF antagonist, ABT-491, which was administered shortly after HI, showed a preventive effect on the axonal dysfunction after HI.

Our study is the first report in the literature which shows that HI causes a partial interruption of the signal through the peripheral nerve fibers and axonal dysfunction, and that these peripheral neuronal changes were reduced by ABT-491, a highly potent and selective PAF antagonist, in a neonatal rat model of HI.

Platelet-activating factor is one of the most potent mediators of inflammatory responses.[17] During hypoxia, ischemia and in other pathologic conditions involving oxidative stress, PAF concentration increases and it becomes a pro-inflammatory messenger and a mediator of neurotoxicity.[11] Excessive PAF promotes neuronal damage and PAF-receptor antagonists elicit neuroprotection in various models of neural injury.[18]

In the last decade, an increased proportion of basic science research has been directed towards evaluating mechanisms and treatment involving cell injury. It has been also shown that use of PAF antagonists after ischemia and reperfusion improves the status of various organs following ischemia in animal models. Administration of PAF antagonists led to a reduction of the ischemia-reperfu-

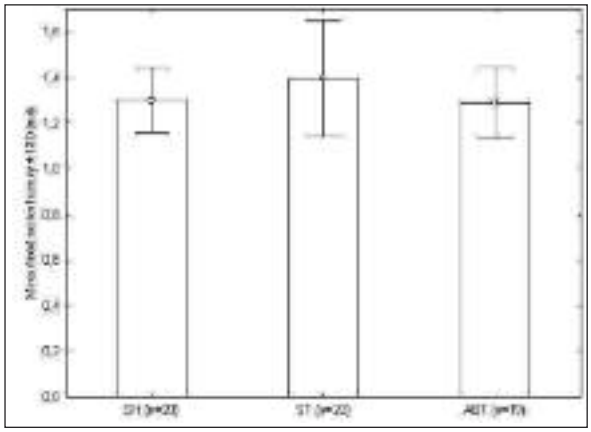

FIGURE 6: The calculated means (with standard deviation-SD) for distal motor latency (DML) of sham (SH), saline treated (ST) and ABT-491 treated (ABT) group. DML values did not show statistically significant differences (p>0.05) among all groups.

sion injury to the heart muscle,[19] a protection against ischemic injury to the kidney,[20] a reduction of the ischemia-reperfusion damage to the lung,[21] and an amelioration of the ischemia-reperfusion injury to the liver.[22] These results have led us to attempt to treatment by a PAF antagonist ABT-491 in models of HI.

The mechanism underlying ABT-491 neuroprotection is not known. However, recent studies have emphasized the anti-apoptotic effect of PAF antagonists and the protective effect of PAF antagonists against apoptotic changes in several tissues and cells.[12,23]

Peripheral nerves are well-vascularized structures that are perfused through a delicate extrinsic and intrinsic microvascular system. Impulse transmission and axonal transport depend on a continuous local energy supply provided by the intraneural microcirculation. Therefore, depletion of high-energy phosphates and resultant conduction failure ensue as soon as intraneural blood flow decreases.[24]

The endoneurial microenvironment of peripheral nerves requires close regulation for normal peripheral nerve function. In human nerves, this is achieved by two specialized interfaces. These are the blood-nerve barrier (BNB) formed by endoneurial microvessels, and the perineurium. The true interface between the blood circulation and endoneurium occurs at the BNB. The human BNB would utilize active transport mechanisms to facilitate the entry of essential polar nutrients and exit of unwanted waste material from the endoneurium. The BNB needs to actively respond to axonal or myelin injury within the endoneurium, contributing to reparative processes. Drugs administered for peripheral nerve disorders have to significantly overcome the BNB in order to efficaciously reach their therapeutic targets.[25] Kvist et al.[26] reported that the neuroprotective action of immunosuppressant tacrolimus in rat peripheral nerve injuries involves nerve regeneration by increasing the rate of axonal regeneration, early restoration of the blood-nerve barrier and limiting the extent of motor end plate loss. In addition, they stated that tacrolimus improved functional recovery of denervated targets by increasing both regenerative and collateral reinnervation in axotomy models, thereby limiting permanent functional loss.

Morphologically, ischemic nerves reveal various pathological abnormalities, including demyelination and remyelination, axonal degeneration and regeneration, focal, multifocal, or diffuse loss of nerve fibers, and endoneural edema.[27] These pathological abnormalities occur not only as a result of trauma and related events but also in compression injuries, entrapments, tourniquet-induced peripheral nerve injuries and acute or chronic hypoxemia.[28] Schmelzer et al.[29] indicated that severe ischemia of peripheral nerve results in reperfusion injury, conduction block, and blood-nerve barrier disruption.

In conclusion, this study suggests that HI has an injury influence on the peripheral nerve and caused axonal degeneration and probably, motor end plate loss, and that platelet-activating factor antagonist, ABT-491, with its neuronal apoptosis reducing effect, may have a preventive effect on the axonal dysfunction after hypoxic ischemia. In addition, the regeneration process by ABT-491 may involve axonal regeneration, restoration of the blood-nerve barrier and limitation the extent of motor end plate loss. More research is needed to fully understand the BNB functions during HI and the effects of ABT-491 on BNB restoration.

Preventing axonal dysfunction together with brain injury may be more effective in the treatment of perinatal HI. However, it is too early to say whether this agent can be used to protect the neurotoxicity of hypoxic ischemia in human beings until further data has been accumulated.

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