FK506 (tacrolimus) inhibits hypoxic ischemic encephalopathy (HIE) in the newborn rat: Evaluation of dose dependency and therapeutic time window

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Abstract

The neuroprotective effects of tacrolimus (FK506) in cerebral infarct adult animal models have been reported in many studies. However there are few studies on neonatal hypoxic-ischemic encephalopathy (HIE) models. The purposes of the present study were to investigate the neuroprotective effects, dose dependency, and therapeutic time window of FK506 using neonatal rat hypoxia ischemia (HI)/reperfusion brain damage models. In seven-day-old Wistar rats (n=211), HI was induced by transient left carotid artery occlusion for 180 min, and 90 min of 8% O₂. The rats were then resuscitated by releasing the carotid artery occlusion and reoxygenated in room air. FK506 (dissolved in 0.1 ml/kg normal saline) or vehicle (0.1 ml/kg normal saline) were intraperitoneally given after the HI insult. In the experiment for dose dependency, 2 mg/kg FK506-treated rats showed significant decreases in the brain damage scores, compared with the vehicle, while there were no significant differences in the 1 mg/kg FK506 group, compared with the vehicle. The neuron survived rates of the 2 mg/kg FK506-treated animals were also significantly higher than those in the vehicle and in the 1 mg/kg FK506 in hippocampus, cerebral cortex and basal ganglia. In the experiment for the therapeutic time window, the brain damage scores in the 2 mg/kg FK506-treated rats were significantly lower than those in vehicle immediately (0 min) and 30 min after HI insult, but not after 60 and 120 min. 2 mg/kg FK506-treated rats demonstrated significantly higher neuron survival rates in hippocampus; immediately (0 min) and 30 min after insult, in the cerebral cortex; immediately (0 min), 30 min and 60 min after insult, in basal ganglia; immediately (0 min) and 60 min after insult, compared with in the vehicle. In the current study, FK506 exerted a neuroprotective effect in a neonatal model with HIE and revealed the existence of a therapeutic time window in FK506 therapy. The findings obtained in the study indicate the possibility of the clinical application of FK506 for the neonatal HIE with indeterminate time of onset.

Introduction

The neonatal mortality rate is decreasing thanks to improvements in neonatal intensive care. However, with no therapies yet established for hypoxic-ischemic encephalopathy (HIE) accompanying neonatal asphyxia, HIE still remains a major cause for neonatal deaths and neurological prognosis.

The pathophysiology of neuronal cell death is being gradually elucidated and various pharmacotherapeutic strategies have been proposed based on the pathophysiology. For example, such pharmacotherapeutic strategies include glutamate receptor antagonists, which are excitatory amino acids, NO synthetase inhibitors against NO neurotoxicity, free radical scavengers against active oxygen and the administration of a neurotrophic factor with the objective of encouraging ischemic tolerance. However, there are no pharmacotherapies that...
have been proven effective in the treatment of HIE in neonate animal experiments. On the other hand, it has been indicated in neonate animal experiments that hypothermia therapy is effective for protecting the brain of patients with HIE\(^2\)\(^-\)\(^4\). A hypothermia therapy to protect the brain of patients with human neonatal asphyxia was, however, revealed to be limited in a multicenter study\(^5\). Side effects of hypothermic therapy itself on neonates, indicate many problems for the application of hypothermia therapy to actual clinical situations.

An immunosuppressive agent, tacrolimus (FK506), is used clinically to inhibit the rejection of transplanted organs in a wide variety of patients including infants. The immunosuppressive mechanism is thought to derive from suppression of T lymphocyte activation resulting from the suppression of calcineurin activation by a complex formed between FK506 and its binding protein (FKBP), i.e. the FK506-FKBP complex\(^6\). In 1994, Sharkey et al. reported that the neuroprotective efficacy of FK506 in a rat cerebral infarction model\(^7\). They suggested that FK506 inhibits nNOS activity and free radical production through the inhibition of calcineurin activity by the FK506-FKBP complex and that FK506 exerts neuroprotective efficacy by inhibiting calcium channels. However, its neuroprotection mechanism involved many unknown factors. Thereafter, the neuroprotective efficacy of FK506 has been reported in many animal adult cerebral infarction models\(^8\)\(^-\)\(^14\). However, few studies have discussed the efficacy of FK506 in an immature brain model with HIE. As for efforts towards the clinical application of FK506 for the purpose of cerebral protection in the treatment of HIE, no studies have been conducted on the dose dependency and the time between the onset of hypoxia-ischemia (HI) and the initiation of therapy, namely the so-called therapeutic time window.

Accordingly, this study aimed to histologically investigate the neuroprotective effect of FK506; to examine the dose dependency of FK506, which is important for clinical application; and to examine the presence of the therapeutic time window in a neonatal rat model with HI/reperfusion brain damage.

**Materials and methods**

1. **Neonatal HIE model preparation**

Wistar dams and their litters were purchased from CLEA Japan Inc (Tokyo, Japan) and maintained on a 12-h cycle of light and dark with food and water freely available. Seven-day-old rat pups of either sex (\(n = 211\)), weighing 15.1 g, were removed from the litters for preparation and study, and returned to be suckled by their dams at all other time during experimental procedures. All experimental procedures were carried out in strict accordance with the guidelines of the Animal Ethical Committee of Tokyo Medical University.

We adopted HI/reperfusion brain insult for the neonatal HIE animal models. Pups were anesthetized with isoflurane inhalation, and the left carotid artery was accessed through a ventral midline cervical incision and transiently occluded with Sugita aneurism clips (Mizuho Co., Ltd, Tokyo, Japan) for 180 min and the neck incision was closed. The entire surgical procedure lasted no longer than 10 min. The rats were then held in an airtight 500 ml plastic chamber through which humidified 8% oxygen/92% nitrogen gas flowed for 90 min. After the HI insult, the rats were then resuscitated by releasing the carotid occlusion and reoxygenating with room air. Rectal temperatures of all animals in the chamber were continuously maintained at 36.5 to 37.0°C automatically by means of a temperature controller system (ATC-101B, Unique Medical Co. Tokyo, Japan) to avoid hypothermia throughout the experiment. The rats were returned to their dams for stabilization for at least 60 min after the surgery and before the period of hypoxia.

2. **Chemicals**

FK506 was purchased from Fujisawa Pharmaceutical (Osaka, Japan). Activated caspase-3 antibody was purchased from Cell Signaling Inc. (Danvers, MA USA).

3. **Experimental protocol**

**Dose dependency**

The neonatal HIE animals (\(n = 97\)) were prepared to elucidate the neuroprotective effects of FK506 and its dose dependency. After the HI/reperfusion insult, rats were divided into three groups. In the first group rats were intraperitoneally randomly given either the 1.0 mg/kg FK506 (dissolved in 0.1 ml/kg normal saline, \(n = 18\)) or the same amount of vehicle (0.1 ml/kg normal saline, \(n = 23\)). In the second group, either 2.0 mg/kg FK506 (\(n = 23\)) or vehicle (\(n = 23\)) was randomly given. The third group (\(n = 10\)) were sham-operated. After drug administration, all animals were then returned to their dams to be cared for and suckled for 24 h.

**Therapeutic time window**

The neonatal HIE animals models (\(n = 160\)) were divided into four groups according to the time for FK506 received after the HI/reperfusion insult to investigate the existence of its therapeutic time window. In each group, rats were randomly given either 2 mg/kg FK506 (dissolved in 0.1 ml/kg normal saline) or the same amount of vehicle (0.1 ml/kg normal saline) intraperitoneally immediately (0 min) (FK506 group \(n = 23\)/vehicles \(n = 23\)), at 30 min (FK506 group \(n = 19\)/vehicles \(n = 18\)), 60 min (FK506 group \(n = 19\)/vehicles \(n = 19\)), or 120 min (FK506 group \(n = 19\)/vehicles \(n = 20\)) after the insult. After drug administration, all
animals were then returned to their dams to be cared for and suckled for 24 h.

4. Pathology
At 24 hrs after the HI insult, all animals were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for histological examination of their brains under deep anesthesia with isoflurane inhalation. The brains were immediately removed intact and then postfixed in the same paraformaldehyde solution for one week. The brains were then embedded in paraffin and cut coronally into 4 µm thick slices. All sections were stained with hematoxylin-eosin (HE) and activated caspase-3.

Sections for activated caspase-3 immunohistochemistry were deparaffinized and hydrated, followed by washing twice in distilled water and in PBS for 5 min. For antigen unmasking, sections were heated in 10 mM sodium citrate buffer (pH 6.0) for 10 min to keep slides fully in buffer and maintain temperature at or just below boiling. Following cooling and washing, the sections were incubated in blocking solution (5% goat serum in PBS) for 60 min to block nonspecific binding. Blocking solutions were removed and the diluted (1 : 200 in PBS) primary caspase-3 antibody was added for incubating overnight at 4°C. Secondary antibody, diluted in blocking solution, was added to each section to incubate for 30 min at room temperature. Following further washes, the sections were incubated with ABC reagents (Vectastain ABC kit, Vector Laboratories Inc. Burlingame, CA, USA) for 30 min at room temperature, washed and reacted with DAB reagent (0.025% diaminobenzidine tetrahydrochloride) and 0.05% H2O2 in Tris-buffered solution for staining. Counterstaining was performed with hematoxylin.

Neuron survival rates
The severity of neuronal damage in the hippocampus, cortex and basal ganglia of coronal section at the level of the arcuate nucleus was evaluated by the numbers of surviving neurons (neuron survival rate), which were counted by investigators who did not know the experimental procedure, under a light microscope at 400 times magnification. The neuron survival rates (%) \( \frac{[\text{the number of intact neurons in lesioned (left) hemisphere}]}{[\text{the number of intact neurons in nonlesioned (right) hemisphere}] \times 100} \) were evaluated.

5. Statistical analysis
The Mann-Whitney U-test was used to examine the brain damage score between the drug received and the vehicle groups. Comparisons between the neuron surviving rates in two groups were made by means of Student’s unpaired t-test, when the data was normally distributed; otherwise the Mann-Whitney U-test was used. The ANOVA, followed by Fisher’s protected least significant difference (PLSD) post-hoc test, was performed to compare the values among more than three groups. A P-value less than 0.05 was considered to indicate a statistically significant difference. The results were expressed as means and SD.

Results
1. Pathology
Fig.1 shows typical histological photomicrographs (400 magnification) of HE stain (a, b, c) and activated caspase-3 immunohistochemistry (d, e, f) in cerebral hemispheres including hippocampus, cerebral cortex and basal ganglia in the vehicle-treated (a, d), the FK506-treated (b, e) of 24 hours after the HI insult, and the sham-operated (c, f) rats. Figure 2 also indicates typical histological photomicrographs (100 magnification) of HE stain in hippocampus (a, b, c), cerebral cortex (d, e, f) and basal ganglia (g, h, i) in the vehicle-treated (a, d, g), the FK506-treated (b, e, h) of 24 hours after HI insult, and the sham-operated (c, f, i) rats. There were pyknotic nuclei, eosinophilic changes, karyorrhexis, neuronal loss, spongy degeneration, and/or cystic changes with macrophages in the brains of vehicle groups (Fig. 1-a, Fig. 2-a, d, g). The immunohistochemical results in the vehicle group also indicated that many activated caspase-3-positive cells, stained dark brown, were observed in the brain (Fig. 1-d). However, in the FK506-treated group, HE stain (Fig. 1-b, Fig. 2-b, c, e, h) and activated caspase-3 immunohistochemistry (Fig. 1-c) were inhibited, suggesting less brain damage. In sham-operated animals, no neuronal damage was detected (Fig. 1-c, f, Fig. 2-c, f, i).

2. Dose dependency
Though there were no significant differences in the brain damage scores in the 1 mg/kg FK506 group, compared with the vehicle group \( (p=0.29) \), 2 mg/kg FK506 group indicated the significant decreases in the brain damage scores, compared with the vehicle group \( (p=0.037) \) (Fig. 3).

The neuron survived rate of the 2 mg/kg FK506-treated group was also significantly higher than those in the vehicle-treated and the 1 mg/kg FK506-treated animals in hippocampus \( (p=0.032, 0.022) \), cerebral cortex \( (p=0.0073, 0.015) \), and basal ganglia \( (p=0.042, 0.023) \).

3. Therapeutic time window
The brain damage scores in the 2 mg/kg FK506-treated group were significantly lower than those in vehicle-treated animals immediately (0 min) and 30 min after HI insult \( (p=0.037, 0.0056) \). However, 60 and 120 min after HI insult, there were no significant differences in them between the two groups \( (p=0.20, 0.53) \) (Fig. 5).

The neuron survived rates in hippocampus (Fig. 6-a) of the 2 mg/kg FK506-treated group were significantly higher immediately (0 min) \( (p=0.037) \) and 30 min \( (p=0.0056) \) after HI insult than those in the vehicle-treated,
but not 60 and 120 min after \( p=0.086, 0.085 \). In cerebral cortex (Fig. 6-b), 2 mg/kg FK506-treated animals indicated significantly higher neuron survived rates immediately (0 min) \( p=0.0092 \), 30 min \( p=0.048 \) and 60 min \( p=0.041 \), but not 120 min \( p=0.55 \), after HI insult. The 2 mg/kg FK506 administration also demonstrated significantly higher neuron surviving rates in basal ganglia (Fig. 6-c) immediately (0 min) \( p=0.0046 \) and 60 min \( p=0.045 \), but not 30 and 120 min \( p=0.17, 0.38 \), after HI insult.

**Discussion**

In the study conducted using brain damage scores to assess neurologic disorders in the entire brain, a significant neuroprotective effect was seen after the administration of FK506 at a dose of 2 mg/kg in our neonatal HIE models, while 1 mg/kg FK506 gave no significant neuroprotection. In addition, in the site-specific assessment of neuron survival rates in the hippocampus, cortex and basal ganglia, 2 mg/kg FK506 administration exerted a more significant neuroprotective effect in all parts of the cerebral cortex, hippocampus and basal ganglia, compared with 1 mg/kg FK506 administration, with no differences seen among the sites. The above findings indicated that 2 mg/kg FK506 significantly inhibits cerebral damage in neonates with HIE dose-dependently.

It is considered that hypoxia-ischemia and later occurred cerebral reperfusion induce various molecular biological mechanisms for neuronal cell death resulting in cerebral disorders associated with HIE. Among these mechanisms, dephosphorylation of various enzymes induced by calcineurin activation is considered to be involved in neuronal damages. It has been reported that calcineurin induces neurotoxicity by activating the NMDA receptor and the release of NO resulting from nNOS activation. In addition, it has been found that apoptosis is induced directly by calcineurin itself through its dephosphorylation of Bcl-x/Bcl-2-associated death promotor homolog (Bad) proteins. Thus, calcineurin is directly and indirectly involved in the onset of neuronal cell death. Although the exact neuroprotective mechanisms have not yet been elucidated, the FK506-FKBP complex may inhibit nNOS production by inhibiting calcineurin activity, resulting in inhibition of apoptosis induced by activation of Bad proteins. In addition, Uchino et al. measured calcineurin activity and reported that calcineurin activity increased 24 hours after ischemia and was inhibited by FK506 or cyclosporin A (CsA). It is also demonstrated in recent years that FK506 reduces oxygen radicals and NO produced by microglia in neurons after ischemia/reperfusion and inhibits the generation of cytotoxic cytokines.

Though many reports have been published on the assessment of the neuroprotective effect of FK506 in studies in adult cerebral infarction models, very few reports have been published that assessed the neuroprotection of FK506 in immature brains. Shibazaki et al. reported, in their study in which neonatal rats born by cesarean operation from a mother with the uterine...
artery temporarily ligated were administered FK506 and measured for their mitochondria respiration and energy metabolism, that lowering of mitochondrial respiratory metabolism and energy metabolism was significantly inhibited in the FK506 treatment group\(^ {21}\). But their study was not in vivo but in vitro. In addition, Nakata et al. demonstrated in their study in vivo using the same neonatal HIE models as ours that the single-agent use of 1 mg/kg FK506 administration did not exert a cerebral protective effect, while the combination with 1 mg/kg FK506 and 7-nitroindazole, a selective nNOS inhibitor, produced a significant neuroprotective effect\(^ {22}\). The current in vivo experiment in the neonatal rat HIE model revealed that even a single administration of FK506 at a higher dose after HI/reperfusion insult protects the brain against HIE.

Fig. 2 Typical histological photomicrographs (100 magnification) of HE stain in the hippocampus (a, b, c), cerebral cortex (d, e, f) and basal ganglia (g, h, i) in the vehicle-treated (a, d, g), the FK506-treated (b, e, h) of 24 hours after HI insult, and the sham-operated (c, f, i) rats.

The dose required for FK506 to exert a significant neuroprotection in the neonatal HIE rats in our study was 2 mg/kg, higher than those reported effective in the adult cerebral infarction model (0.3 mg/kg to 1.0 mg/kg)\(^ {7\sim 10, 23, 24}\). The difference in effective doses between the neonate and the adult model may be related to the immaturity of neonatal brains in the developmental stage. Another possible reason for the difference is that the current model was loaded with hypoxia in addition to ischemia, with the objective of making the model closer to the clinical features of HIE, while the adult cerebral infarction model was loaded with only ischemia. Under the loading of hypoxia in addition to ischemia, a higher dose of FK506 may have been needed to maintain the effective blood concentration after reperfusion. In addition, consideration should be also given to the possibility that the insult of hypoxia-ischemia followed by reperfusion may induce stronger and larger brain damage than mere ischemia.

Although the onset of HIE was occurred before and during birth, clinical intervention can be started only postnatally. Therefore, there is a time lag between the onset of injury and the start of cerebral protection. Accordingly, the presence/absence of a time difference...
between the beginning of HIE injury and the initiation of effective neuronal intervention, namely the therapeutic time window, is extremely important in clinical terms. The recent studies using phosphorus and proton magnetic resonance spectroscopy (MRS) in both encephalopathic infants\(^{29}\) and a neonatal piglet model\(^{30}\) demonstrated a brief period of apparently normal cerebral energy metabolism after severe hypoxia-ischemia, followed by a progressive decline in cerebral energy metabolism. This latent phase after resuscitation represents an opportunity for the therapeutic intervention, i.e. a “therapeutic time window”. In the examination of the presence/absence of the therapeutic time window in the FK506 single administration through the assessment of the entire brain by the brain damage scores, the cerebral protective effect of FK506 was observed in administration of the drug immediately and 30 minutes after resuscitation, whereas it was not observed at 60 minutes after resuscitation. The above findings indicated that there is a therapeutic time window of at least 30
Fig. 5  The brain damage scores in the experiment for therapeutic time window
The 2 mg/kg FK506-treated group showed a significantly lower score by the Mann-Whitney U-test than those in vehicle-treated animals immediately (0 min) and 30 min after HI insult ($p=0.037$, 0.0056). However, 60 and 120 min after HI insult, there were no significant differences in them between the two groups ($p=0.20$, 0.53).
minutes in the FK506 treatment in the assessment of the entire brain.

As previously discussed, there are several neuro-protective mechanisms of FK506, which have not been clearly elucidated yet, but the action is considered to be expressed at different time points during the process of neuronal cell death. The nNOS activated cells, supposed to be inhibited by FK506, are expressed early within three hours after hypoxia-ischemia is loaded. On the other hand, microglia, inactivated early within 2 hours after ischemia/reperfusion and activated 5 hours after ischemia/reperfusion, is considered to be one of the causes for delayed neuronal cell death. Li et al. reported that FK506 inhibited dephosphorylation of Bad 3 hours after reperfusion and decreased activity of caspase-3, which is thought to play a central role in apoptotic pathway, 24 hours after reperfusion in a cerebral ischemic mouse model. Bud is an important proapoptotic member of the Bcl-2 family, which is dephosphorylated by calcineurin and promotes apoptotic pathway including caspase activation. Therefore, FK506 is thought to inhibit caspase activity by inhibition of calcineurin activity. In addition, Manabat et al. concluded, in their study in which caspase-3 activities expressed in the apoptosis pathway were measured in the neonatal rat model with transient cerebral ischemia, that the expression of caspase-3 was not seen until three hours but seen at eight hours after the insult. Therefore, cerebral neuronal injury caused by apoptosis is considered to be a delayed neuronal cell death. We speculate that the presence of the therapeutic time window in the FK506 therapy in the present study is caused by the fact that FK506 may act against the pathogenesis of those late-onset neuronal damage.

We also examined the regional specificity of the therapeutic time window. The tissue-specific therapeutic time windows were 60 minutes or more in the cerebral cortex, 30 minutes or more in the hippocampus and 0 minutes in the basal ganglia. We strictly assessed the neuron survived rates in basal ganglia to interpret its therapeutic time window as 0 minutes, because the significance was not confirmed at 30 minutes after the insult in spite of the significance at 60 minutes. Therefore, we supposed that there may be cerebral tissue-specific specificity in the therapeutic time window of FK506. Previous investigations using an adult rat cerebral infarction model demonstrated that both apoptosis and necrosis were seen in a large number of cells in the caudate nucleus and putamen, whereas in the cerebral cortex apoptosis was seen in a larger number of cells than necrosis. They also reported that FK506 administration significantly reduced apoptosis in the cerebral cortex. Bredt et al. showed that the number of nNOS-positive cells was larger in the cerebral cortex.
and smaller in the basal ganglia with the striatum as a central component. The regional differences in the therapeutic time window of FK506 in the brain may originate from the site-specific specificity in the distribution of apoptosis passway, nNOS and calcineurin positive cells.

**Conclusion**

In a neonatal rat HI/reperfusion model, we observed a neuroprotective effect of FK506 at 24hours after insult. We also confirmed its dose dependency and therapeutic time window. The findings obtained in the current study indicate the possibility of the clinical application of FK506 to neonatal HIE.

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**References**


新生仔ラットモデルに対する FK506 (tacrolims) の低酸素性虚血性脳症抑制効果；用量依存性、Therapeutic time window の検討

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【要旨】近年、免疫抑制剤 FK506 が成人脳梗塞動物モデルにおいて脳保護効果を示すとする報告が散見される。しかし、低酸素性虚血性脳症の新生仔動物モデルの報告はほとんどみられない。そこで、我々は新生仔ラットを用いて低酸素-虚血/再灌流モデルを作成し、FK506 の脳保護効果および用量依存性と therapeutic time window について検討した。対象は日齢 7 の Wister 系ラット、左総頭動脈をクリップで一過性に閉塞し、チャンバー内を 90 分間 8% 酸素に保ち、低酸素虚血 (HI) 負荷とした。180 分後に左総頭動脈閉塞を解除し、room air にて蘇生した。

用量依存性：HI 負荷後の薬剤投与量により、ラットを 3 つのグループに分けた (FK506 2 mg/kg；n = 23, 1 mg/kg；n = 18, serine；n = 23)。

24 時間後の病理組織学的評価で FK506 2 mg/kg 投与群のみ有意な神経保護効果を認めた。

therapeutic time window：ラットを HI 負荷後から薬剤投与 (FK506 2 mg/kg) までの時間で ① 直後の薬剤投与群、② 30 分後投与群、③ 60 分後投与群、④ 120 分後投与群の 4 グループに分けた。

HI 負荷後 24 時間後に病理組織学的に評価した。脳全脳を評価した brain damage score では 0 分、30 分後投与では有意な脳保護効果を示した。

さらに海馬、大脳皮質、基底核でそれぞれ個別に生存細胞数の割合を調べたところ、大脳皮質は、60 分後投与においても有意な神経保護効果を示した。

今回我々は、新生仔 HI ラットモデルにおいて、FK506 の脳神経保護効果に用量依存性が存在することを確認した。さらに HI 負荷から薬剤投与までの間に therapeutic time window が存在することから、臨床応用への可能性が示唆された。

＜キーワード＞FK506 (tacrolims)、神経保護、用量依存性、Therapeutic time window、新生仔ラット