The neuroprotective effects of long-term, low dose administration of FK506 (tacrolimus) on hypoxic–ischemic encephalopathy in the neonatal rat

Makiko MIYATA, Yukito TAKEI, Akinori HOSHIKA

Department of Pediatrics, Tokyo Medical University

Abstract

FK506 (tacrolimus) is an immunosuppressant agent used in organ transplantation. We previously reported that single doses of FK506 were ineffective after hypoxia–ischemia (HI). We investigated whether low dose (1 mg/kg) FK506 shows protective effects, if given repeatedly over a longer period of time after insult. We also studied the brain protection mechanism of FK506 by studying the dynamics of activated microglia, myelin, and temporal changes of the brain derived neurotrophic factor (BDNF) level, and whether the above procedure produces less side effects, by examining the mortality and body weight increment rates. In 7-day-old Wistar rat pups (n=69), HI was induced by transient occlusion of the left carotid artery and 8% O₂ for 90 min. The rats were then resuscitated by releasing the carotid artery occlusion and reoxygenated in room air. After the HI/reperfusion insult, rats were randomized to a group receiving intraperitoneal 1.0 mg/kg FK506 dissolved in 0.1 ml/kg normal saline or the same amount of vehicle (0.1 ml/kg normal saline) for one time on the first day (FK506–1d, n=13; vehicle–1d, n=10), or once daily for 3 days (FK506–3d, n=13; vehicle–3d, n=13), or once daily for 7 days (FK506–7d, n=7; vehicle–7d, n=13). At 8 days after the HI/reperfusion insult, the ratio of the left (with lesion) to the right (without lesion) cerebral hemispheric weight in the FK506–3d and FK506–7d rats were significantly higher than those in the vehicle–3d and vehicle–7d (p=0.002, p=0.02, respectively). The histological findings of Iba-1 and MBP stains demonstrated that low dose FK506 administrations for 3 (FK506–3d rats) or 7 days (FK506–7d rats) inhibited the activation of microglia and myelin sheath damage in the damaged brain, compared with the vehicle rats. BDNF levels in the FK506–3d and FK506–7d rats were significantly higher than those in the vehicle–3d and vehicle–7d (p=0.05, p=0.04, respectively). There was no significant difference between these parameters in the FK506–1d rats, compared with those in the vehicle–1d group. The weight gains during the experiment for 7 days in the FK506–3d and the FK506–7d rats were significantly lower than vehicle–7d. The mortality rates in the FK506–7d rats were significantly higher than the vehicle–7d rats. These results demonstrated that the low dose FK506, single dose of which was not effective, was neuroprotective for brain damage, when administered for longer terms in neonatal HIE animal models. The inhibition of activated microglia and the elevation of neurotrophic factors have been subjected to be involved in the mechanism of the neuroprotective effects of long-term low dose FK506 administration on the neonatal HIE. Even at low doses, FK506 had more influence on the general condition of neonatal animals, when it was given for longer periods than when given in single doses.
Introduction

Hypoxic–ischemic encephalopathy (HIE), caused by neonatal asphyxia can have serious sequelae such as neonatal death, cerebral palsy, and mental retardation to name a few\(^{13,9}\). Much research and treatment studies have been aimed at the conquest of this problem\(^2\), but so far no established solution has been found. In 1992, some neuroprotective effects of an immunosuppressant, cyclosporine A (CsA) in a localized brain ischemic model in adult animals were reported for the first time\(^9\). This was followed by another study, which reported that another immunosuppressant, FK506 also possessed similar neuroprotective effects\(^7\). FK506 has been clinically used as an immunosuppressant frequently after many surgical transplant procedures, including neonatal cases\(^9\,\,\,10\). It also can be used to protect the brain through peripheral intravenous injection because of its high osmosis through the blood–brain barrier\(^11\,\,\,13\).

These two features would make FK506 clinically useful for human neonates, should its brain protection effects be confirmed. To attain this goal, our research group first studied neuroprotective effects by FK506, using a neonatal hypoxia–ischemia (HI)/reperfusion brain injury model in the rat\(^5\), and Sunohara et al showed dose dependent therapeutic effects\(^5\). They demonstrated that while a higher dose (2 mg/kg) had protective effects, a lower dose (1 mg/kg) did not. Likewise, using a similar model, Nakada et al found neuroprotective effects of combined administration of low dose FK506 and 7NI for 7 days\(^5\). They, however, also reported some FK506-related side effects such as retarded body weight increase, and deaths during the treatment. These findings suggest a reciprocal dose dependency tendency of FK506 on the neonatal HIE, i.e., higher dosage would produce greater brain protection effects through suppressed immunity, but at the same time, more severe side effects. We noted that not only necrosis but also apoptosis, in other words, delayed neuronal cell death, were involved in the brain injury due to HIE in the neonate. Therefore, we hypothesized that neuroprotective effects without such side effects might be provided by long-term administration of a lower dose of FK506, single doses of which are not effective, for the prevention of the further progression of delayed neuronal cell death which would be seen several days after the HI insult. The beneficial mechanism of the FK506 is attributed to its neuronal protection effects by inhibitory action on the calcineurine, which is done by making a conjugation with its binding protein immunophillin, FKBP12\(^16\,\,\,17\). Recently, several investigators speculated that FK506 with FKBP52, another immunophillin, would facilitate nerve regeneration by stimulating the production of neural nutrition factors\(^19\).

Furthermore, the increased number of the activated microglial cells also accounted for the death of neurons as well as the white matter lesion\(^19\,\,\,21\). Namely, these microglial cells facilitate production of neurotoxic factors such as cytokines which in turn cause neuronal death and destruction of the oligodendrocytes, which eventually induces white matter lesions\(^22\,\,\,28\). On the other hand, these activated microglia protect the brain by producing many neuroprotective substances such as neurotrophic factors\(^20\,\,\,22\). Therefore, the activated microglia have a double-edged sword effect by possessing neuroprotective and neuroinjurious features. However, although some brain protective effects of FK506 have been demonstrated, its mechanisms, especially on the microglia, and on neurotrophic factors are not well understood.

The purposes of the present paper are to investigate using HI/reperfusion neonatal rat models: (1) whether a low dose (mg/kg) FK506 (no protective effects have been reported with a single dose after HI exposure) shows some protective effects if given repeatedly over a longer period of time, (2) whether it is possible to elucidate the operating brain protection mechanism of FK506 used with the above procedure by studying the dynamics of activated microglia, myelin, and temporal changes of the BDNF level, (3) whether the above procedure produces less side effects by examining the mortality and body weight increment rates.

Materials and methods

Subjects

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 \(n=\) 69, weighing between 12 and 18 grams, were removed from the litters for preparation and study, and returned to be suckled by their dams at all other times during the experimental procedures.

Experimental procedure

All experimental procedures were carried out in strict accordance with the guidelines of the Animal Ethical Committee of Tokyo Medical University. In neonatal asphyxia, temporal hypoxia–ischemia is always followed by reperfusion. Therefore, we adopted a brain insult of not only HI but also reperfusion for the neonatal HIE animal models. Under 5% isoﬂurane inhalation anesthesia, the left carotid artery was exposed, and temporarily occluded by clamping with the Sugita Aneurysm Clip (Mizuno Co. Ltd, Tokyo, Japan) for 90 min and the neck incision was closed. The entire surgical procedure lasted no longer than 10 min. The rats were then placed in an air tight 500 ml plastic chamber containing humidified 8% oxygen/92% nitrogen gas, creating the

---
desired HI condition for 90 min. The rats were then resuscitated by releasing the carotid occlusion and reoxygenned 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.

Chemicals

FK506 was purchased from Fujisawa Pharmaceutical (Osaka, Japan).

Experimental protocol

After the HI/reperfusion insult, rats were divided into three groups, according to the periods of drug administration. In the first group, rats were intraperitoneally randomly given either 1.0 mg/kg FK506 (dissolved in 0.1 ml/kg normal saline, n = 13) or the same amount of vehicle (0.1 ml/kg normal saline, n = 10) after the insult for one time on the first day (one dose). In the second group, either 1.0 mg/kg FK506 (n = 13) or vehicle (n = 13) was randomly given every 24 hrs for three days (total 3 doses). In the third group either 1.0 mg/kg FK506 (n = 7) or vehicle (n = 13) was randomly given every 24 hrs for seven days (total 7 doses). After drug administration, all animals were then returned to their dams to be cared for and suckled for seven days.

Brain weight measurement and histological assessment

On the eighth day after the HI insult (pups were then 15 days old), circulating blood was taken transcardially under deep anesthesia with isoflurane inhalation. The brains were immediately removed intact and then cut to divide them into left and right hemispheres and each weight was measured. In order to assess the severity of the whole brain damage, we measured the hemispheric brain weight to obtain the ratio of left (with lesion) and right (without lesion) hemispheric brain weight. Then the cerebral hemispheres were placed into 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for one week. The brains were then embedded in paraffin and cut coronally into 4 μm thick slices. All coronal sections were stained with ionized calcium-binding adapter molecule 1 (Iba-1), and myelin basic protein (MBP).

Iba-1 staining

After deparaffination, the sections were place in the autoclave (105°C, 10 min) with citric acid buffer (pH = 6.0, 0.01 mol). In order to block endogenous peroxidase activity, rehydrated sections were treated with 0.3% H2O2 in absolute ethanol for 10 min, then washed twice in PBS for 5 min. Then polyclonal Anti Iba-1, Rabbit (Wako Pure Chemicals, Osaka, Japan) was added and the sections were incubated overnight at 4°C. After the incubation, the staining took place using the LSAB kit (Dako Cytomation Co. Ltd, Denmark) as a secondary antibody. The method indicated by the instructions of the manufacture was followed completely. For contrast, standard hematoxylin staining was used. In order to assess the influence of FK506 on activated microglia after HI/reperfusion brain injury, both the left (with lesion) and the right (without lesion) hemispheres of all coronal sections at the level of the arcuate nucleus were photographed by a CCD camera (Olympus Co., Tokyo, Japan) to measure the areas of the Iba-1 positive portion using an image analyzer system (Scion image, Scion Corporation, Washington, D.C., U.S.A.). The ratio of the left and the right Iba-1-positive areas in the hemispheres were assessed.

Myelin Basic Protein staining (MBP)

Sections were deparaffinized and rehydrated, followed by washing twice in distilled water and in PBS for 5 min. To block endogenous peroxidase activity, rehydrated sections were treated with 0.3% H2O2 in absolute ethanol for 10 min, then washed twice in PBS for 5 min. Then polyclonal antibody (Nichirei Biosciences Inc, Tokyo, Japan) was added to each section, which was incubated overnight at 4°C. After washing with PBS, sections were processed by the Histofine Simple Stain Max PO(M) (Nichirei Biosciences Inc). The manufacturer's instructions were followed exactly. Counterstaining was performed with the standard hematoxylin method. In order to assess the influences of FK506 on the myelin sheath damages after HI/reperfusion brain injury, both hemispheres with (left) and without (right) lesions of all coronal sections at the level of the arcuate nucleus were photographed by a CCD camera (Olympus Co., Tokyo, Japan) to measure the areas of the MBP-positive portion using an image analyzer system (Scion image). The ratios of the left and the right Iba1-positive hemispheric areas were assessed.

Measurement of the Brain derived neurotrophic factor (BDNF)

The plasma was separated by centrifugation with EDTA for 10 min at 800 G, then preserved at −50°C, then BDNF were extracted by an enzyme–linked immunosorbent assay kit (ChemKine BDNF Sandwich ELISA Kit, Chemicon International Co, USA) later.

Body weight gain and mortality

We also evaluated nutritional status and mortality rates during the 8-day experiment. The body weight just before the HI procedure (age 7 days) and euthanization (15–days old), were measured and the ratio between these two measurements was calculated for every pup.

5. Statistical analysis

Comparisons between values in the 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. Fisher’s exact test was used to compare the mortality rates. A value of p<0.05 was
considered to indicate a statistically significant difference. The results were expressed as means±SD.

Results

There was no significant difference in body weight between the FK506 and vehicle treated rats before HI insult.

Cerebral hemispheric weight

There was no significant difference between the ratio of the left (with lesion) to right (without lesion) cerebral hemispheres in the FK506–1d group and that in the vehicle–1d group. The ratios in the FK506–3d and FK506–7d groups were significantly higher than those in the vehicle–3d and vehicle–7d (p=0.002, p=0.02, respectively), which means that FK506 administration for 3 or 7 days significantly lowered the severity of the brain damages compared with the vehicle (Fig. 1).

Iba–1 staining

Fig. 2 shows typical histological examples of anti–Iba–1 antibody staining of the cerebral hemisphere in the vehicle (Fig. 2a), and the FK506 (Fig. 2b) rats. There were some degrees of increases in the intensity of staining as well as in the number of stained microglia, which means activated microglia, in the left (with lesion) hemisphere of all rats, especially in the vehicle rat (Fig. 2a).

We assessed the ratio of the left (with lesion) to right (without lesion) hemispheric Iba–1–positive areas in order to evaluate the activation of microglia due to brain damage. There was no significant difference between the ratio in the FK506–1d group and that in the vehicle–1d group (Fig. 3). However, the ratios in the FK506–3d and FK506–7d rats were significantly lower than those in the vehicle–3d and vehicle–7d (p=0.001, p=0.03, respectively) (Fig. 3), which means that FK506 administrations for 3 or 7 days showed to inhibit the activation of microglia in the brain, compared with the vehicle.

MBP staining

Fig. 4 shows typical histological examples of MBP staining of cerebral hemisphere in the vehicle (Fig. 4a, c, e), and the FK506 (Fig. 4b, d, f) rats. There were some degrees of decreases in MBP staining, which means myelin sheath damage after HI/reperfusion brain injury, in the left (with lesion) hemispheres of all rats, especially in the vehicle rats (Fig. 4a, c, e).

We assessed the ratio of left (with lesion) to right (without lesion) hemispheric MBP–positive area in order to evaluate myelin sheath damage. There was no significant difference between the ratio of the FK506–1d and the vehicle–1d rats (Fig. 5). However, in the FK506–3d and FK506–7d, the ratios were significantly higher than those in the vehicle–3d and vehicle–7d (p=0.04, p=0.02, respectively) (Fig. 5), which means that FK506 administration for 3 or 7 days caused less myelin sheath damages in the brain than the vehicle alone.

Brain derived neurotrophic factor (BDNF)

The BDNF levels in the FK506–3d and FK506–7d rats were significantly higher than those in the vehicle–3d and vehicle–7d (p=0.05, p=0.04, respectively) (Fig. 6), whereas there was no significant difference between

---

**Fig. 1** The ratio of the left to right cerebral hemispheric weight. There was no significant difference in FK506–1d and vehicle groups. The ratio in the FK506–3d (p=0.002), the FK506–7d (p=0.02) were significantly higher than in the vehicle–only animals. It is assumed that the higher the value of ratio, the lesser the brain damage.

**Fig. 2** Typical examples of Iba–1 staining of the left hemisphere (with lesion) in the vehicle–7 (a), and the FK506–7 (b).
The ratio in the FK506-1d and that in the vehicle-1d (Fig. 6).

**Body weight gain and mortality rates**

There was no significant difference between the weight gains in the FK506-1d and the vehicle-1d (Fig. 7), though in the FK506-3d and FK506-7d, these were significantly higher than those in the vehicle-3d and vehicle-7d ($p=0.005$, $p=0.002$, respectively) (Fig. 7), which means deterioration in the nutritional status in the rats of FK506 administrations for 3 or 7 days.

No control or FK506-1d animal died at any stage of the experiment. The mortality for FK506-3d was 19% (3/16), whereas for FK506-7d, it was 36% (4/11). The difference between FK506-3d and vehicle-3d was not significant ($p>0.01$), but there was a significant
Fig. 5 The ratio of the left to the right hemispheric MBP staining positive area. There was no significant difference in the FK506-1d and that in the vehicle. The ratio in the FK506-3d ($p=0.001$), and the FK506-7d ($p=0.03$) were significantly higher than the vehicles. It is assumed that the higher the ratio, the lesser the damage to the myelin sheath.

Fig. 6 The blood levels of BDNF. There was no significant difference in the FK506-1d and vehicle groups. The values in the FK506-3d ($p=0.05$) and FK506-7d ($p=0.04$) were significantly higher than the vehicles.

Fig. 7 The body weight gains. There was no significant difference between the weight gains in the FK506-1d and the vehicle-1d. In FK506-3d and FK506-7d, the ratios were significantly lower than in the vehicle group ($p=0.005$, $p=0.002$, respectively).

Table 1 Mortality rates.

<table>
<thead>
<tr>
<th>Dosage</th>
<th>Survived, n (pts (%))</th>
<th>Died, n (pts (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>FK506-1d</td>
<td>FK506-3d</td>
</tr>
<tr>
<td>1</td>
<td>13, (100%)</td>
<td>13, (81%)</td>
</tr>
</tbody>
</table>

In the FK506-1d, all 13 animals survived. The difference between FK506-3d and vehicle-3d was not statistically significant ($p>0.1$), there was a difference between FK506-7d and vehicle-7d ($p=0.02$).
white matter lesions or their prevention, although neonatal white matter lesions, such as in periventricular leukomalacia causes cerebral palsy, have close relationships with neurological sequelae.

Recently, some investigators reported that FK506 exhibits its protective effects by directly controlling the activation and population of the microglia which leads to the inhibition of the cytokines such as IL-1β, and TNFα production. The multiplication of the activated microglia is connected with the injury of the oligodendrocytes, which induces the myelin sheath damage, which in turn causes lower white matter volume. Therefore, the FK506 is likely to reduce white matter damages associated with the volume decrement through its inhibitory action on the activation of the microglia.

The production of neurotrophic factor was increased to protect nerve cells on brain injury. The increment of neurotrophic factors in the CSF and/or plasma has been also reported in the cases of the neonatal HI. One possible explanation is that some immunosuppressant inhibit calcineurin, which in turn indirectly inhibits the dephosphoration of the cyclic AMP–response element–binding protein (CREB), one of the cAMPs of the neurotrophic factor BDNF. This series of actions promotes production of the BDNF. Also, there is a report in which the FK506, together with its binding protein immunophilin, FKBP52, acts directly on this neurotrophic factor in an adult Parkinson's animal model. Compared with the controls, the experimental groups which received low- and long–term doses of FK506 showed higher BDNF levels, which suggest that the desired brain protection effects were obtained by facilitating production of BDNF, a neurotrophic factor.

The systemic influence of this medication must be considered. Compared to the 1-day group, higher numbers of nutritional disturbances and deaths were found in the 3-day and 7-day groups, particularly in the latter. FK506 has both dose dependent neuroprotective and the immunosuppressant effects. Thus, it is expected that extended FK506 usage would lead to more desirable neuroprotective effects but at the same time, more undesirable immunosuppressant disturbances as side effects. This expectation was borne out in the present study. Among the three groups, the 3-day group showed neuroprotective effects and relatively mild side effects. Observations of cerebral energy metabolism in the newborn piglet's HIE models using magnetic resonance spectroscopy demonstrated that they had not only an acute ("primary") depleted brain energy metabolism during HI insult but also delayed ("secondary") energy failure within a few days after the insult. Perhaps, the administration of FK506 continued for three days might be long enough to maintain its blood level to somewhat prevent this delayed ("secondary") energy failure. Finally, considering the long-term nerve damaging processes such as apoptosis, more investigations for using FK506 for a longer time are desirable.

**Conclusion**

In the present study, neuroprotective effects of FK506, an immunosuppressant drug were demonstrated on a neonatal HI/reperfusion model. When this agent was administered in a long-term low dose mode (1 mg/kg/day for 3 or 7 days), it was more effective than when it was given in a single low dose mode (1 mg/kg/day for 1 time). The study also suggested that the brain protective effects of FK506 seen after HI/reperfusion injury, involved the inhibition of activated microglial cells and myelin sheath injury, as well as the facilitation of production of the neurotrophic factor BDNF. However, it was found that even such low dose administration still caused some side effects, such as elevated mortality rate and decelerated body weight increment rate due to the growth, when it was used for certain extended periods. For these reasons, it is important to find an optimal mode of administration, which maintains sufficient therapeutic brain protection effects, while minimizing adverse reactions.

**Acknowledgments**

The authors thank Prof. Kudo, Mr. Ishikawa, and Mr. Fujita, Department of Pathology, Tokyo Medical University, for useful technical advices for histology. The authors are indebted to Prof. J. Patrick, Barron of the International Medical Communications Center of Tokyo Medical University for his review of this manuscript.

This study was supported by MEXT, Kakenhi (13670840).

**References**

5) Hagberg H, Gilland E, Diener NH : Hypoxia–ischemia in the neonatal rat brain: histopathology after post-treatment with NMDA and non–NMDA


33) Sharkey J, Jones PA, McCarter JF, Kelly JS: Calcineurin inhibitors as neuroprotectants. CNS...
新生仔ラット低酸素虚血/再灌流脳障害モデルに対する
免疫抑制剤 FK506 低用量長期間投与の神経保護効果

宮 田 真貴子 武 井 章 人 星 加 明 徳
東京医科大学小児科学講座

今回我々は、新生仔ラット低酸素虚血/再灌流脳障害モデルを用いて、低酸素性虚血性脳症 (HIE) に対する免疫抑制剤 FK506 の神経保護効果について検討した。本研究の目的は、(1) 単独では神経保護効果を示さない低用量 (1 mg/kg) FK506 を長期間投与することで神経保護効果を得ることができるかどうか、(2) ミクログリアの活性化、ミエリン鞘および神経栄養因子 BDNF に及ぼす影響について検討することで FK506 低用量長期間投与の神経保護作用機序を説明できるかどうか、(3) 体重増加率と死亡率を検討し、FK506 低用量長期間投与がより副作用の少ない治療法かどうかを検討することである。対象は、日齢 7 の Wistar 系ラット (n=69)、左脳動脈をクリップで一過性に閉塞し、シャンバー内を 8% O2 に保ち 90 分の低酸素虚血 (HI) 負荷とした。90 分後に閉塞を解除し、room air で蘇生した。FK506 単日投与群は荷負解除直後に FK506 (1 mg/kg) を生食に溶解し 1 回腹腔内投与した。3 日投与群は同様の操作を 24 時間毎に計 3 回投与し、7 日投与群は 24 時間毎に計 7 回投与した。それぞれの群で同量の生食を同様回数腹腔内投与し、各群の対照とした。HI 負荷後 8 日目に血液採取、脳摘出、脳組織の免疫染色および体重測定を行った。左右大脳半球重量比の比較では、FK506 の 3 日、7 日投与群は生食群に比べて有意に脳重量の減少を抑制した。Iba-1 染色と MBP 染色の検討では、FK506 の 3 日、7 日投与群は生食群に比べて、ミクログリアの活性化及びミエリン障害を有意に抑制した。また神経栄養因子 BDNF の血中濃度は、FK506 の 3、7 日投与群は生食群に比べて有意に上昇していた。1 日投与群ではこれらについて FK506 群と生食群の間に有意な差を認めなかった。一方、体重増加率は FK506 の 3 日、7 日投与が生食群に比べ有意に低く、また FK506 の 7 日投与群は死亡率が有意に高かった。これらの結果より新生児 HIE モデルにおいて低用量 FK506 は単日投与では神経保護効果を得られないが、長期間投与することで神経保護効果を得られることがわかった。また FK506 低用量長期間投与による神経保護作用機序には、活性化ミクログリアとミエリン障害の抑制、および BDNF 産生促進が関与することが推測された。しかし FK506 は副作用の観点から低用量であっても長期間投与することで全身に与える影響も強いか示された。

＜キーワード＞ 低酸素性虚血性脳症 (HIE)、FK506、活性化ミクログリア、白質障害、神経栄養因子 BDNF