Combination therapy with FK506 and 7-nitroindazol inhibits brain damage due to hypoxia-ischemia/reperfusion in the newborn rat

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Abstract

The aim of this study was to examine neuroprotective effects of the combination therapy with FK506 and 7-nitroindazole (7NI) using the neonatal rat hypoxia ischemia/reperfusion (HI) brain damage model. In seven-day-old Wistar rat pups (n = 190), HI was induced by transient left carotid artery occlusion for 180 min, followed by 90 min of 8% O2. The rats were then resuscitated by releasing the carotid artery occlusion and reoxygenated in room air. In the first experiment, the rats (n = 107) were divided into three groups according to the drugs received after HI. In the first group (n = 38), the animals were given the combined administration of 1.0 mg/kg FK506 plus 50 mg/kg 7NI (FK506 + 7NI, n = 18), or vehicle (n = 20). In the second group (n = 33), 1.0 mg/kg FK506 only (only FK506, n = 17), or vehicle (n = 16). In the third group (n = 36), 50 mg/kg 7NI only (only 7NI, n = 18) or vehicle (n = 18). In this study, the combination therapy (FK506 + 7NI) showed significantly neuroprotective effect at 24 h after HI, by histopathological analysis (p < 0.05), neither of which was effective when used alone (only FK506, only 7NI) at these doses. In the second experiment, the animals (n = 83) were randomly divided into three groups according to the periods of the combined administration of 1 mg/kg FK506 with 50 mg/kg 7NI. The [FK506 + 7NI]-1d group (n = 26) was given one time after HI insult on the first day. The [FK506 + 7NI]-7d group (n = 28) was given the same doses of FK506 and 7NI after HI insult and every 24 hrs for 7 days (total 7 times) following the onset of HI insult. The vehicle group (n = 29) was given the same amount of saline and peanut oil. On 8 days after HI, histopathological analysis showed that the decreasing rates of hemispheric area in the [FK506 + 7NI]-1d group and [FK506 + 7NI]-7d group were significantly lower than in the vehicle group (p = 0.0332, p = 0.0277). There was no significant difference in the rates between the [FK506 + 7NI]-1d and the [FK506 + 7NI]-7d groups. The weight gains during the experiment for 7 days in the [FK506 + 7NI]-1d was significantly higher (p < 0.01), and the mortality rate of the [FK506 + 7NI]-1d group was significantly lower than the [FK506 + 7NI]-7d groups (p < 0.01). In this study, we demonstrated the neuroprotective effects of combination therapy with FK506 and 7NI against neonatal HI brain damage models.

Introduction

Hypoxic ischemic encephalopathy after neonatal asphyxia is associated with poor neurological prognosis, and is an important cause of newborn death. No effective treatment, however, has been established for this condition. In hypoxic ischemic encephalopathy, neuronal energy deficiency due to hypoxia first triggers a steep increase in glutamic acid level, inducing NMDA receptor activity and various cascade processes, which lead to excessive Ca2+ influx into cells and the production of cytotoxic substances, including reactive oxygen...
species (ROS) and nitric oxide (NO)\textsuperscript{12,20}.

Recently, the immunosuppressant FK506, which has been shown to ameliorate ischemic neuronal death caused by disorders such as adult cerebral infarction\textsuperscript{20}, has been a focus of research. FK506 targets calcineurin, a phosphatase known to control calcium influx into cells under physiological conditions, mediated by the IP3 receptor, NMDA receptor and ion channels\textsuperscript{39}. Under hypoxic conditions, activated calcineurin accelerates neuronal apoptosis through the mechanisms including dephosphorylation and activation of the NMDA receptor, increase in both glutamic acid release and calcium ion influx into cells, inactivation of various neurotransmitters\textsuperscript{40}, mitochondrial dysfunction and nitric oxide synthase (NOS) activation\textsuperscript{39}. It has been recently reported that calcineurin dephosphorylates BAD protein and directly induces apoptosis\textsuperscript{39}.

The topic of the present study, however, the effect of FK506 on newborn animals, has not been previously reported elsewhere. However, little is known about the neuroprotective effect of FK506 against neonatal hypoxic ischemic encephalopathy, and furthermore, we must consider systemic immunosuppressive side effects of the drug on neonates.

On the other hand, NO has been demonstrated to play a crucial role in neonatal hypoxic ischemic encephalopathy\textsuperscript{12,17}. The exact process of neuronal death caused by NO has not been clarified, but it is assumed that NO is produced along with excitatory amino acids and other substances when intracellular calcium concentrations increase after hypoxic ischemia (HI), and that NO hampers the ATP synthetic ability of intracellular mitochondria or becomes peroxynitrite through reaction with superoxide anions, thus inducing cell damage\textsuperscript{39,40}. Moreover, recent research has shown that NO has both neuropathic and neuroprotective effects in ischemic neuropathy, depending on the different isoforms of NOS involved in its synthesis\textsuperscript{10–13}. One of the isoforms, neural nitric oxide synthase (nNOS), which is involved in the neurotoxic effects of glutamatic acid, is up-regulated in cerebral ischemia earlier than other isoforms, and has a peak concentration between 10 minutes to 3 hours after onset of ischemia\textsuperscript{21}. nNOS is known to be activated by a rapid increase in intracellular concentrations of calcium ions, after which it synthesizes a large amount of NO, thus exacerbating neuronal damage\textsuperscript{39}.

In the present study, we focused on a selective nNOS inhibitor, 7-nitroindazole (7NI). 7NI is considered to exert its neuroprotective effect by lowering the peak NO level during both hypoxic ischemia and reperfusion\textsuperscript{14}. In studies using adult animal models, 7NI reduced infarct size in various models, including those of global ischemia\textsuperscript{13}, transient global ischemia\textsuperscript{14,17} and transient focal ischemia\textsuperscript{18}. In all of these studies, however, 7NI was administered prior to HI, which is different from the timing of administration in the clinical setting. Some reports on neonatal rats exist, but in these reports 7NI monotherapy was observed to be effective only when it was administered prior to HI\textsuperscript{14,19,20}, and most reports indicated that 7NI alone, when administered after HI, was not effective\textsuperscript{20}. Although the neuroprotective effect of 7NI is considered to be dose-dependent\textsuperscript{21}, it appears that the dosage needs to be kept low because a high dose possibly induces inductive nitric oxide synthase (iNOS)\textsuperscript{22} and increases the probability of adverse reactions.

It is expected that the combination therapy of FK506 and 7NI, neither of which was effective when used alone in low dose, is neuroprotective for hypoxic ischemic encephalopathy of neonate.

The purposes of the present study was to investigate the neuroprotective effects and the efficacy of combination therapy of FK506 and 7NI, compared with administration of FK506 or 7NI alone, using neonatal rats concomitantly after HI/reperfusion.

**Materials and methods**

1. **Animal 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 = 190), weighing 10.8–17.8 g, were removed from the litters for preparation and study, and returned to be suckled by their dams at other time. All experimental procedures were carried out in strict accordance with the guidelines of the Animal Ethical Committee of Tokyo Medical University. The HI animal model, based on Rice’s procedure\textsuperscript{26}, was performed. Pups were anesthetized with isoflurane inhalation, and the left carotid artery was accessed through 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. 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.
2. Materials

FK506 and 7NI were purchased from Fujisawa Pharmaceutical (Osaka, Japan) and Sigma Chemical Co. (St. Louis, MO), respectively.

3. Experimental protocol

Experiment 1.

After the HI insult, the animals \( n = 107 \) were divided into three groups according to the drugs received. Animals were intra-peritoneally given FK506 (dissolved in 0.1 ml/kg normal saline), 7NI (dissolved in 0.1 ml/kg peanut oil), or both with the same amount of vehicles (0.1 ml/kg normal saline and 0.1 ml/kg peanut oil). In the first group \( n = 38 \), the animals were randomly given either combined administration of 1.0 mg/kg FK506 plus 50 mg/kg 7NI (FK506 + 7NI) or vehicle \( n = 20 \). In the second group \( n = 33 \), the animals were randomly given either the FK506 only (only FK506, \( n = 17 \)) or vehicle \( n = 16 \). In the third group \( n = 36 \), the animals were randomly given either the 7NI only (only 7NI, \( n = 18 \)) or vehicle \( n = 18 \). Animals \( n = 5 \) were sham-operated.

After drug administration, all animals were then returned to their dam to be cared for and suckled for 24 h.

Experiment 2.

After the above HI insult, the animals \( n = 83 \) were randomly divided into three groups according to the periods of the combined administration of FK506 with 7NI. These groups received combination of FK506 with 7NI (1.0 mg/kg FK506, dissolved in 0.1 ml/kg normal saline; 50 mg/kg 7NI, dissolved in 0.1 ml/kg peanut oil) or vehicle (0.1 ml/kg normal saline and 0.1 ml/kg peanut oil) treatment once a day for 7 days at 3, 24, 48, 72, 96, 120, 144 hrs following the HI insult. In the first group (the [FK506 + 7NI]-1d group, \( n = 26 \)), the animals were given both FK506 and 7NI (FK506, 1.0 mg/kg, dissolved in 0.1 ml/kg normal saline; 7NI, 50 mg/kg with 0.1 ml/kg peanut oil) one time after HI insult on the first day, followed by 0.1 ml/kg normal saline and 0.1 ml/kg peanut oil every 24 hours for 7 days intraperitoneally. In the second group (the [FK506 + 7NI]-7d group, \( n = 28 \)), the animals were given the same amount of both drugs (FK506, 1.0 mg/kg, dissolved in 0.1 ml/kg normal saline; 7NI, 50 mg/kg with 0.1 ml/kg peanut oil) after HI insult and every 24 hrs for 7 days (total 7 times) intra-peritoneally. In the third group (vehicle group, \( n = 29 \)), the animals were given both normal saline 0.1 ml/kg and 0.1 ml/kg ml peanut oil after the HI insult and every 24 hrs for 7 days (total 7 times) intraperitoneally. Animals \( n = 5 \) were sham-operated.

After drug administrations, all animals were then returned to their dam to be cared for and suckled for 7 days.

4. Pathology

Experiment 1.

At 24 hrs after the HI insult, all animals were perfused transcardially with a 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.

Activated caspase-3 antibody was purchased from Cell Signaling Inc. (Danvers, USA). Sections 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 washes, 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 (Vectorstain 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.

We examined the coronal sections of the hippocampus, cortex, and basal ganglia at the level of the arcuate nucleus under a light microscope. HI insult in the 7 day-old rats in our experiment induced combination of the following neuronal damages; pyknosis, eosinophilic change, karyorrhexis, loss of neurons, caspase-3-positive cells, spongy degeneration, and/or cystic change with macrophages in the hippocampus, frontal and temporal cortices, and/or basal ganglia (see Fig. 1). The severity of brain damage was estimated by the extent of the damaged neurons in the brain by two observers who were blinded to the drug treatment, using the following scores: 0, no damage; 1, neuronal damages are observed in only one cerebral tissue among the tissues (hippocampus, frontal and temporal cortices, or basal ganglia); 2, neuronal damage is observed in two cerebral tissues as mentioned above; 3, neuronal damage is observed in three cerebral tissues as mentioned above; 4, spongy degeneration and/or cystic change with macrophages is observed in the coronal section.
neuron surviving rates.

The severity of neuronal damages 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 surviving rates), which were counted by investigators who did not know the experimental procedure, under a light microscope at 400 times magnification. The neuron surviving rates (\%) \( \left( \frac{\text{[the number of intact neurons in lesioned (left) hemisphere]} \times 100}{\text{[the number of intact neurons in non-lesioned (right) hemisphere]}} \right) \) were evaluated.
Experiment 2.

Seven days after the HI insult, all animals were treated in the same way as experiment 1 to obtain the brains samples, which were also embedded in paraffin and cut coronally into 4 μm thick slices. All coronal sections were stained with hematoxylin-eosin (HE).

Decreasing rates of hemispheric area (%)

In order to assess the severity of brain damage after 7 days HI insult, both lesioned (left) and nonlesioned (right) hemispheres of all coronal sections at the level of the arcuate nucleus were photographed by a CCD camera (Olympus Co., Tokyo, Japan). The areas of lesioned and nonlesioned hemispheres were measured using an image analyzer system (NIH image). Decreasing rates of hemispheric area (%) [(area of nonlesioned − area of lesioned) ÷ area of nonlesioned] were assessed among three groups. We also evaluated mortality rates and nutritional status during the experiment for 7 days [weight gain (g) = weight on the 7th day − weight on the first day].

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 values in two groups were made by means of an unpaired t-test, when the data was normally distributed; otherwise the Mann-Whitney U-test was used. Comparisons between values in more than three groups were made by means of analysis of variance (ANOVA), followed by the Fisher’s protected least significant difference (PLSD) post-hoc test. Fisher’s exact test was used to compare the mortality rates. A value of p < 0.05 was considered statistically significant. The results were expressed as means and SD.

Results

1. Experiment 1

Figure 1 shows typical histological examples of HE stain (a, c, e, g, i) and activated caspase-3 immunohistochemistry (b, d, f, h, j) in hippocampus (a-j), cortex and basal ganglia for the FK506 + 7NI (c, d), and the vehicle (a, b), the only FK506 (e, f), the only 7NI (g, h) of 24 hours after HI insult, and sham-operated (i, j) rats. There are pyknotic nuclei (the nuclei which are
Fig. 2  a) The brain damage scores. Brain damage in the FK506+7NI group was significantly less 24 after the HI insult, compared with the vehicle group ($p<0.05$). There were no significant differences between the only FK506 and the only 7NI groups, compared with the vehicle groups.  b) The neuron survival ratio. In the FK506+7NI group (hatched bars), the neuron survival rates in hippocampus ($p=0.062$), cortex ($p=0.035$), and basal ganglia ($p=0.0346$), were significantly higher than in the vehicles (close bars).

Fig. 3  Histological findings (HE stain) of (a) vehicle, (b) [FK506+7NI]-1d, (c) [FK506+7NI]-7d group, and (d) sham-operated.

shrunken and dense), eosinophilic changes, karyorrhexis, neuronal loss, spongy degeneration, and/or cystic changes with macrophages in the hippocampus (Fig. 1-a), frontal cortex (histological examples is not shown), and basal ganglia (histological examples is not shown) in the three vehicle-treated groups. The immunohistochemical results in the three vehicle-treated also indicated that many activated caspase-3-positive cells, stained

( 6 )
dark brown, were observed in the brain (Fig. 1-b). However, in the FK506 + 7NI group, HE stain (Fig. 1-c) and activated caspase-3 immunohistochemistry (Fig. 1-d) were inhibited suggesting less brain damage. In sham-operated animals, no neuronal damage was detected (Fig. 1-i, j).

The brain damage scores in the FK506 + 7NI group were significantly decreased 24 after the HI insult, compared with the vehicle group (p < 0.05). However, there were no significant differences in those in the only FK506 and the only 7NI groups, compared with the vehicle groups (Fig. 2-a).

In the FK506 + 7NI group, the neuron surviving rates in hippocampus (p = 0.0062), cortex (p = 0.0035), and basal ganglia (p = 0.0346), were significantly higher than in the vehicles (Fig. 2-b).

2. Experiment 2

Figure 3 shows typical histological examples of HE stain in the vehicle (Fig. 3-a), the [FK506 + 7NI]-1d (Fig. 3-b), the [FK506 + 7NI]-7d group (Fig. 3-c), and sham-operated (Fig. 3-d) rats. In the vehicle group, the construction is extensively collapsed and the lesioned hemisphere is remarkably atrophic (Fig. 3-a). In the [FK506 + 7NI]-1d group (Fig. 3-b) and the [FK506 + 7NI]-7d group (Fig. 3-c), there is very slight atrophy in the lesioned hemisphere, but the structure is maintained.

The decreasing rates of hemispheric area in the [FK506 + 7NI]-1d group and [FK506 + 7NI]-7d group were significantly lower than that in the vehicle group (p = 0.0332, p = 0.0277). There was no significant difference in the rates between the [FK506 + 7NI]-1d group and the [FK506 + 7NI]-7d groups (Fig. 4).

The weight gains during the experiment for 7 days in the [FK506 + 7NI]-1d group and [FK506 + 7NI]-7d group were significantly lower than that in the vehicle group (p < 0.001, p < 0.01). The weight gains in the [FK506 + 7NI]-1d group was significantly higher than that of the [FK506 + 7NI]-7d group (p < 0.01) (Fig. 5-a).

Mortality rates

The rate of mortality of the [FK506 + 7NI]-7d group was significantly higher than that of the [FK506 + 7NI]-1d group (p = 0.452) [Fig. 5-b].

Discussion

In a neonatal rat hypoxia ischemia/reperfusion model, we observed a neuroprotective effect at 24 h after insult, of concomitant use of FK506 (1 mg/kg) and 7NI (50 mg/kg), neither of which are effective alone at these doses. In contrast to previous studies in neonatal animals, which used an artery occlusion model, we used a hypoxia ischemia/reperfusion model. Moreover, we administered drugs after HI and still achieved a neuroprotective effect.

We speculate that the neuroprotective effect produced by the combination therapy was a result of simultaneous interruption of two sets of reactions in multiple processes leading to neuronal damage after hypoxic ischemia.

In the processes leading to neuronal damage after cerebral ischemia, nNOS is activated at an early stage7 through the NMDA receptor12, which controls neuronal damage, and is dependent on increased glutamic acid concentrations after cerebral ischemia, and inducing a large volume of NO, leading to neuronal damage during ischemia. In previous studies, ischemic neuronal damage was significantly reduced in nNOS knock-out mice using either an adult MCAO model12 or a neonatal HI model20, therefore, the importance of nNOS inhibition in the treatment of ischemic neuronal damage is clear.

7NI is a selective nNOS inhibitor20, which, according to Higuchi et al.19, produces a neuroprotective effect by lowering two peak levels of NO, one during ischemia and another during reperfusion. There are an increasing number of reports on the neuroprotective effect of 7NI in neonatal models19,20. Among these studies, however, a neuroprotective effect was observed only in the studies in which 7NI was administered prior to HI19,20, and relatively high doses (50–100 mg/kg in Higuchi et al., 75–150 mg/kg in Muramatsu et al., and 100 mg/kg in Ishida et al) were necessary to achieve efficacy19,20. In the present study, a neuroprotective effect was not obtained in the group treated with 7NI 50
mg/kg alone after HI. Muramatsu et al.\textsuperscript{(19)}, who examined post-HI administration, and noted NOS activity inhibition with high doses of 7NI (75–150 mg/kg), but noted no corresponding neuroprotective effect in histological analysis.

Recent studies have determined that, relative to adult animals, in neonatal animals the neurotoxicity mediated by NMDA receptors tends to be enhanced\textsuperscript{(27)} and the 7NI doses necessary to diminish NOS activity are higher\textsuperscript{(26)}, although the NO production is lower\textsuperscript{(27,28)}. These characteristics of neonatal animals appear to make the treatment of ischemic neuronal damage difficult.

On the other hand, since Sharkey et al.\textsuperscript{(3)} reported the neuroprotective effect of FK506 for ischemic brain damage, a large number of studies have indicated its beneficial effect, including therapeutic efficacy for cerebral infarction and neuroprotective effect in adult animals of local and forebrain ischemia\textsuperscript{(5–29,31)}. It is thought that FK506 forms a complex with FK506 binding protein 12 (FKBP12) and alters the structure of the catalytic site of calcineurin, a protein phosphatase found in T-cells, thus inhibiting its activity and producing a neuroprotective effect\textsuperscript{(32)}, but the exact mechanism of this process has not yet been determined. Although the effect of FK506 against ischemic brain damage has been traditionally attributed to the inhibition of excitotoxic mechanisms including that of NO, since Bucher et al.\textsuperscript{(33)} reported that FK506 doesn't inhibit the excitotoxicity of NMDA and AMPA, it has been increasingly recognized that inhibition of microglia or astrocytes substantially contributes to the neuroprotective effect of FK506\textsuperscript{(34–37)}. Recent studies suggest that microglia are involved in post-ischemic inflammation and exacerbation of neuronal damage, and also they play an important role in the process of delayed neuronal death. In \textit{in vitro} studies, microglia have been shown to produce reactive oxygen species (ROS) and NO\textsuperscript{(38)} and to induce neurotoxic cytokines including FasL and TNF-\alpha, and proinflammatory cytokines including IL-1\beta, IL-6 and INF-\gamma\textsuperscript{(37–40)}. Wakita et al. found that FK506 inhibited astrocytes and microglia and reduced infarct size in a global cerebral ischemia model\textsuperscript{(38)}. In an adult cerebral ischemia model, FK506 elicited a neuroprotective effect even when it was administered two\textsuperscript{(39)} or three hours\textsuperscript{(41)} after ischemia. From these results and knowledge of its target molecules, we can speculate that FK506 is effective against relatively delayed neuronal death in ischemic brain damage. The effect of FK506 in the neonatal model is not clear due to the scarcity of studies on this topic. Shibazaki et al.\textsuperscript{(42)} reported that FK506 administered after ischemia inhibited the deterioration of mitochondrial respiratory metabolism and cerebral energy metabolism in an ischemia model generated by uterine artery occlusion and reperfusion in rats at 17 days of gestation. The FK506 dose used in the present study, 1 mg/kg, has been found to produce a neuroprotective effect in previous studies using adult animal models\textsuperscript{(329–341)} and was also used in the aforementioned study by Shibazaki et al. However, we did not find that FK506 exerted any

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Fig. 5-a}
\end{figure}

\begin{table}[h]
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\begin{tabular}{l|l|l}
\hline
 & the [FK506+7NI]-1d group & the [FK506+7NI]-7d group \\
\hline
the number of deaths & 1 & 4 \\
\hline
the number of survivors & 26 & 24 \\
\hline
total & 27 & 28 \\
\hline
\end{tabular}
\caption{Fig. 5-b}
\end{table}

\textbf{a)} Weight gains during the 7-day experiment. In the [FK506+7NI]-1d group and [FK506+7NI]-7d group weight gains were significantly lower than in the vehicle group ($p < 0.01$, $p < 0.01$). The weight gain in the [FK506+7NI]-1d group was significantly higher than that of the [FK506+7NI]-7d group ($p < 0.001$). \textbf{b)} Mortality rates. The rate of mortality of the [FK506+7NI]-7d group was significantly higher than that of the [FK506+7NI]-1d group ($p = 0.452$).
neuroprotective effect when used alone at this dose. We speculate that the neuroprotective effect produced by the combination therapy was a result of simultaneous interruption of two sets of reactions in multiple processes leading to neuronal damage after hypoxic ischemia. As mentioned earlier, 7NI produces a neuroprotective effect by lowering the peak levels of NO at early stage of hypoxic ischemia, while FK506 inhibits delayed neuronal death in ischemic brain damage. As mentioned earlier, 7NI reduces neuronal damage in the early stage after HI by suppressing nNOS, and FK506 inhibits microglia and astrocytes, thereby reducing delayed damage induced by microglia and inflammatory reactions.

We did not confirm the regional specificity of the neuroprotective effect of combination therapy with FK506 and 7NI, 24 hours after HI on histological assessment in cortex, hippocampus, and basal ganglia. Concerning the regional specificity of the neuroprotective effect of these two drugs, 7NI is reported not to inhibit the NO activity of the striatum. This fact seems to be related to the order of nNOS expression, which is enriched temporarily in the cortex, hippocampus, corpus striatum and thalamus of embryonic and neonatal rat brains. There is no report about the regional peculiarity of the effect of FK506. We speculate that combination therapy succeed to protect the basal ganglia including striatum, thereby FK506 compensates for the weakness of 7NI.

We also confirmed the neuroprotective effect of combination therapy with FK506 and 7NI, at 8 days after HI. 7NI has a short half-life, and is generally considered not to have a tendency to inhibit later stage neuronal damage. Effective treatment of delayed neuronal damage requires high doses. However, in a previous study it was found that 7NI induced iNOS when administered at a high dose, in contrast, FK506 inhibits delayed neuronal death. According to past reports on neuronal damage after ischemia in adult animals, approximately one-third of neuronal damage can be attributed to delayed neuronal death, suggesting that reduction of delayed neuronal death as well as acute treatment is essential in order to achieve a significant therapeutic benefit. We speculate that the positive effect in the present study was achieved because we were able to simultaneously interrupt two different time-points in the complex cascades of reactions leading to neuronal death. Tweel et al. also used combination therapy in a neonatal HI model, but they used a combination of 7NI and aminoguanidine, an iNOS inhibitor, and found that the treatment successfully reduced infarct size. In their study, the combination of two NOS inhibitors produced long-term improvement, but did not inhibit cytokines or inflammatory reaction. FK506 compensated for the inability of 7NI to affect cytokines and inflammation.

Eight days after HI in our study, the rate of body weight gain of the group receiving the combination of FK506 and 7NI (in both the group the [FK506+7NI]-1d group and [FK506+7NI]-7d group) was significantly lower than that of the control group (p<0.01, p<0.01), suggesting that an adverse reaction with respect to nutritional states was occurring. The weight gains in the [FK506+7NI]-1d group was significantly higher than that of the [FK506+7NI]-7d group (p=0.452). Although there was no statistically significant difference in neuroprotective effect between the two treated groups, the results of the [FK506+7NI]-1d treatment group were better than those of the [FK506+7NI]-7d group in the present study. The neuroprotective effects of 7NI and FK506 are considered to be dose-dependent.

In the present study, we did not examine their dose dependency, which needs further investigation. However, for 7NI, it appears that the dosage needs to be kept low because a high dose may induce inducible nitric oxide synthase (iNOS) and increase adverse reactions. Furthermore, FK506 is an immunosuppressant, and is also thought to have huge influences on the systemic conditions to be used for immature neonates at high-dosage. Allowing for the complexity of cascades leading to ischemic neuronal death and the adverse effects of the drugs, it seems unlikely that a clinical neuroprotective effect can be achieved with only one drug. Further studies are needed to develop a more neuroprotective regimen with minimal adverse effects, to refine the timing of combination therapy for neonatal HIE.

Conclusion

In a neonatal rat hypoxia ischemia/reperfusion model, we observed a neuroprotective effect at 24 h after insult, of concomitant use of FK506 (1 mg/kg) and 7NI (50 mg/kg), neither of which are effective alone at these doses. Also we confirmed the neuroprotective effect of combination therapy with FK506 and 7NI at 8 days after HI. There was no significant difference in the neuroprotective effect between the [FK506+7NI]-1d and the [FK506+7NI]-7d groups. The weight gains and the mortality rate during the experiment for 7 days in the [FK506+7NI]-1d was significantly higher and significantly lower respectively than the [FK506+7NI]-7d groups.

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References

新生仔ラット低酸素虚血/再灌流脳障害モデルに対する
FK506, 7-nitroindazole 併用療法の神経保護効果

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【要旨】 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、新生児仮死後の低酸素性虚血性脳障害に対する免疫抑制剤 FK506 と神経型一酸化窒素 (nNOS) 抑制剤 7-nitroindazole (7NI) の併用療法の神経保護効果について検討した。対象は 7 日齢の Wister 系ラット (n=190)、左総頸動脈をクリップで一時的に閉塞し、チャンバー内を 90 分間 8% O2 に保ち、低酸素虚血 (HI) 負荷とした。180 分後に左総頸動脈塞を解除し、room air にて蘇生した。実験 1 では、HI 負荷後薬剤投与により、ラット (n=107) を 3 つのグループに分けた最初のグループ (n=38) は、FK506 1.0 mg/kg + 7NI 50 mg/kg の併用群 (FK506+7NI, n=18) と対照群 (n=20)、2 番目 (n=33) は、FK506 1.0 mg/kg 単独群 (only FK506, n=17) と対照群 (n=16)、3 番目 (n=36) は、7NI 50 mg/kg 単独群 (only 7NI, n=18) と対照群 (n=18) とした。24 時間後の病理組織学的評価で FK506+7NI 群のみ有意な神経保護効果を認めた (p<0.05)。FK506 単独群、7NI 単独群はいずれもこの用法では神経保護効果を認めなかった。実験 2 では、ラット (n=83) を FK506 1 mg/kg と 7NI 50 mg/kg の併用療法の投与期間により 3 群に分けた。[FK506+7NI]-1d 群 (n=26) は、FK506 1 mg/kg と 7NI 50 mg/kg を HI 負荷後当日に 1 回投与した。[FK506+7NI]-7d 群 (n=28) は同用量を HI 負荷後、当日と以後 24 時間おきに 7 日間、計 7 回投与した。対照群 (n=29) は同量の生理食塩水と peanut oil を投与した。HI 負荷後 8 日目に病理組織学的評価を行った。[FK506+7NI]-1d 群と [FK506+7NI]-7d 群では脳側半球の面積減少率が対照群と比較し有意に低かった (p=0.0332, p=0.0277)。同減少率は [FK506+7NI]-1d 群と [FK506+7NI]-7d 群の間では有意差は認めなかった。7 日間の実験期間中の体重増加は [FK506+7NI]-1d 群が [FK506+7NI]-7d 群と比較し有意に高く (p<0.01)、死亡率は [FK506+7NI]-1d 群が [FK506+7NI]-7d 群と比較し有意に低かった (p<0.01)。今回、我々は新生仔 HI モデルで FK506 と 7NI の併用療法の神経保護効果を確認した。

キーワード 低酸素性虚血脳症 (HIE), 7-ニトロインドゾール (7NI), FK506, 神経保護効果