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Protective effect of FK506 (Tacrolimus) in pentylenetetrazol-induced kindling in mice

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Abstract

The repeated administration of otherwise subconvulsant dose of pentylenetetrazol (PTZ) is known to produce chemical kindling in animals. In our study, chronic administration of subconvulsant dose of PTZ (40 mg/kg) produced chemical kindling in mice. Pretreatment with L-arginine (50–100 mg/kg ip) potentiated the PTZ-induced kindling, whereas N^{ω}-nitro-L-arginine methyl ester (L-NAME) (10–20 mg/kg ip) showed a protective effect. FK506, a potent neuroprotective agent, dose dependently (0.5–1 mg/kg po) decreased the kindling score. When given in combination, L-NAME potentiated the protective effect of lower dose of FK506 (0.5 mg/kg) on PTZ-induced kindling. L-Arginine (50–100 mg/kg) reversed the protective effect of FK506 (1 mg/kg) and L-NAME (20 mg/kg). Biochemical studies showed the potential role of free radical toxicity in the kindled mice, as there was an increased lipid peroxidation as indicated by elevated malondialdehyde (MDA) and nitrite levels and decrease in GSH and superoxide dismutase (SOD) levels. FK506 pretreatment significantly reversed the elevated MDA and nitrite levels, GSH and SOD depletion induced by PTZ treatment. In conclusion, the results of the present study suggest the possible neuroprotective action of FK506 against PTZ-induced kindling.

Keywords: Epilepsy; PTZ; Kindling; FK506; Free radicals; Neuroprotection

1. Introduction

Kindling is a chronic model of epilepsy and eleptogenesis. Since its first description by Goddard (1967), several studies have appeared in the literature describing its validity and application in studying the underlying course of epilepsy and testing of antiepileptics. Kindled seizures in animals after daily electrical stimulation of the amygdaloid complex have been described (Takei et al., 1999a). The repeated administration of an initially subconvulsant dose of pentylenetetrazol (PTZ) (a blocker of the Cl^- channel of $GABA_A$ receptors) (Corda et al., 1990), determines the appearance and progressive intensification of convulsant activity, culminating in a generalized seizures (i.e., chemical kindling).

Several evidences demonstrated that excessive production of nitric oxide (NO) could be detrimental for the neuronal cells both in vitro and in vivo (Murphy, 1999). Inhibitors of nitric oxide synthase (NOS), such as N^{ω}-nitro-L-arginine methyl ester (L-NAME), have prevented the development of focal seizures (Cassina and Radi, 1996). The demonstration of early seizure-induced free radicals (OH) formation fulfils the critical requirement in establishing the role of oxygen radicals in the pathophysiology of epilepsy (Christine et al., 1999). Another source of OH radical is the peroxynitrate anion ($ONOO^-$), which is generated by the spontaneous reaction of O_2^- and NO. Under condition of increased NO synthesis, the formation of OH^- is favored strongly, thus initiating the processes of protein and lipid peroxidation that induce cell damage. FK506, a potent neuroprotective agent in vitro and in vivo, increases NOS phosphorylation and decreases NO production (Zhang and Steiner, 1995); thus, the neuroprotective effect of FK506 could be attributed to inhibition of NOS activity (Macleod and Butcher, 2002). Recently, it has been reported that FK506 reduces the formation of free radicals and inhibited lipid peroxidation in a number of tissues (Centikale et al., 1999). The role of oxidative stress in kindling epilepsy is still to be established.

The present study was aimed to explore the role of oxidative stress in the pathophysiology of kindling epilepsy and to study the possible mechanisms in the neuroprotective effect of FK506 in PTZ-induced kindling in mice.

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2. Material and methods

2.1. Animals

Albino mice (Laka strain) of either sex (20–30 g) bred in Central Animal House facility of the Panjab University were used. The animals were housed under standard laboratory conditions, maintained on a natural light and dark cycle and had free access to food and water. Animals were acclimatized to laboratory conditions before the test. Each animal was used only once. All experiments were carried out between 0900 and 1700 h. The experimental protocols were approved by the Institutional Animal Ethics Committee and conducted according to the Indian National Science Academy guidelines for the use and care of experimental animals.

2.2. Chemical kindling in mice

PTZ was given in a subconvulsant dose of 40 mg/kg on alternative days for the period of 12 days in control mice. After each injection of PTZ, occurrence of CNS excitation was noted over 10–15 min by observing the mice in a plexiglass chamber ($30 \times 24 \times 22$ cm) with partitions in between. The intensity of behavioral seizure was evaluated using a four-point scoring system: 0 = no effect; 1 = jerks; 2 = Straub's tail; 3 = clonus; the degree of behavioral response increased progressively over the days until the animal exhibited full motor seizures. Mean kindling score was plotted against the duration of treatment (Gasior et al., 2000).

In order to study the role of FK506 in PTZ-induced kindling epilepsy and its possible mechanism of action, the treatment of FK506 and other combinations with it were given on all the 12 days of treatment in PTZ treated mice. Mean kindling score was calculated of the treated group on the 12th day after 2 h of the last administration of test drug and was compared with the control group receiving only PTZ.

2.3. Dissection and homogenization

On the 12th day of study, the animals were sacrificed by decapitation. The brains removed, rinsed in isotonic saline and weighed. A 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). The postnuclear fraction for enzyme assay was obtained by centrifugation of the homogenate at $12000 \times g$ for 20 min at 4 °C.

2.4. Lipid peroxidation assay

The quantitative measurement of lipid peroxidation in the whole brain was measured according to the method of Wills (1966). The amount of malondialdehyde (MDA) formed was measured by the reaction with thiobarbituric acid at 532 nm using Perkin Elmer lambda 20 spectrophotometer. The results were expressed as nmol of MDA/mg protein using the molar extinction coefficient of chromophore ($1.56 \times 10\ M^{-1}\ cm^{-1}$).

2.5. Estimation of reduced glutathione

Reduced glutathione in the forebrain was estimated according to the method of Ellman (1959). A 0.75 ml of homogenate was precipitated with 0.75 ml of 4% sulfosalicylic acid. The samples were centrifuged at $1200 \times g$ for 15 min at 4 °C. the assay mixture contained 0.5 ml of supernatant and 4.5 ml of 0.01 M DTNB. The yellow color developed was read immediately at 412 nm spectrophotometrically. The results were expressed as nanomole GSH per milligram protein.

2.6. Superoxide dismutase (SOD) assay

SOD activity was assayed according to the method of Kono (1978), wherein the reduction of nitrazoblue tetrazolium (NBT) that was inhibited by SOD is measured at 560 nm spectrophotometrically. Briefly, the reaction was initiated by the addition of hydroxylamine hydrochloride to the reaction mixture containing NBT and postnuclear fraction of the homogenate. The results were expressed as units per milligram protein, where one unit of enzyme is defined as the amount of enzyme inhibiting the rate of a reaction by 50%.

2.7. Protein estimation

The protein content was measured according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.8. Determination of nitrite levels

Nitrite is the stable end product of NO in vitro systems. Accumulation of nitrite was measured in cell-free supernatants from brain homogenate by spectrophotometer assay based on Griess reaction (Raghvendra et al., 2000). Briefly, the supernatant of brain homogenate was mixed with equal volume of Griess reagent (1% sulphanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% H_3PO_4) and incubated at room temperature for 10 min to yield a chromophore. Absorbance was read at 543 nm spectrophotometrically. The nitrite concentration was calculated from a standard curve and expressed as micromolar per milliliter.

2.9. Drugs, sources and treatment

The drugs used in the present study were obtained from the following drug houses: PTZ (Sigma, USA), FK506 (Panacea Biotech Lalru Punjab, India), L-NAME (Sigma) and L-arginine (S.D. Fine chemicals, India). FK506 was

suspended in 0.5% CMC and administered per os (orally). PTZ, L-NAME and L-arginine were dissolved in saline and administered intraperitoneally. FK506 and respective combinations were given on all days except withdrawal and PTZ, which was administered on alternative day of the study. FK506 was administered 1 h before and other drugs were administered 30 min before PTZ. PTZ-treated group received second injection of the vehicle and they also received the injection of the vehicle on the next day, when PTZ was not given.

2.10. Statistical analysis

Results are expressed as mean ± S.E.M. The significance of the difference in the responses of treatment groups in comparison to the control was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test. $P < .05$ was considered statistically significant.

3. Results

3.1. Effect of FK506 on PTZ-induced kindling

Chronic treatment for a period of 12 days with subconvulsive dose of PTZ (40 mg/kg) induced kindling in mice. Pretreatment with FK506 (0.5–1 mg/kg po) for 12 days of the study showed a dose-dependent decrease in the mean kindling score as assessed on the 1st, 3rd, 5th, 7th, 9th and 12th day of the study, respectively (Fig. 1).

3.2. Effect of L-NAME or L-arginine alone, and in combination on PTZ-induced kindling

L-NAME showed protective effect as it dose dependently (10–20 mg/kg ip) decreased the cumulative kindling score. L-Arginine per se (50–100 mg/kg ip) decreased the threshold of PTZ-induced kindling as there was an increase in the mean kindling score as assessed on the 1st, 3rd, 5th, 7th, 9th and 12th day of the study. L-Arginine also reversed the protective effect of L-NAME on PTZ-induced kindling (Fig. 2).

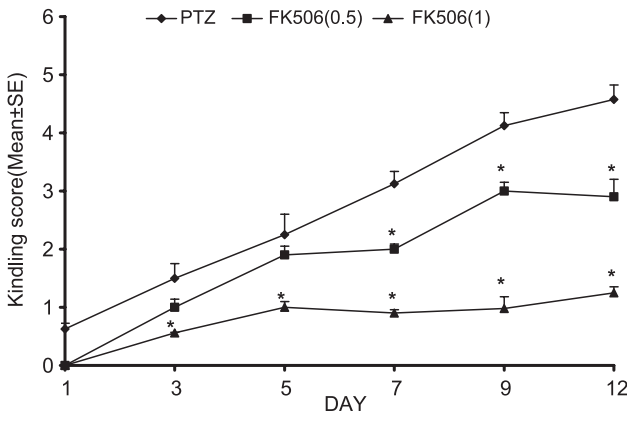

Fig. 1. Protective effect of FK506 (0.5–1 mg/kg po) on PTZ-induced the mean kindling score as assessed on the 1st, 3rd, 7th, 9th and 12th day of the study ($n = 8–14$). * $P < .05$ as compared with control (ANOVA followed by Dunnett's test).

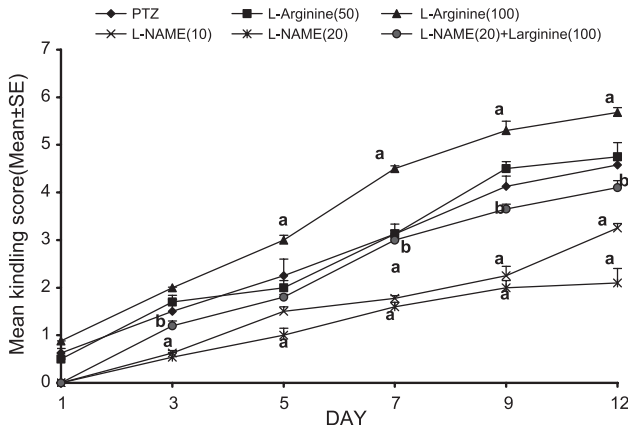

Fig. 2. Effect of L-NAME (10–20 mg/kg ip) and L-arginine (50–100 mg/kg ip) on PTZ-induced kindling (kindling score (mean ± SE), n=8–14). [a] $P < .05$ as compared with PTZ-treated group (ANOVA followed by Dunnett's test). [b] $P < .05$ as compared with L-NAME-treated group (ANOVA followed by Dunnett's test).

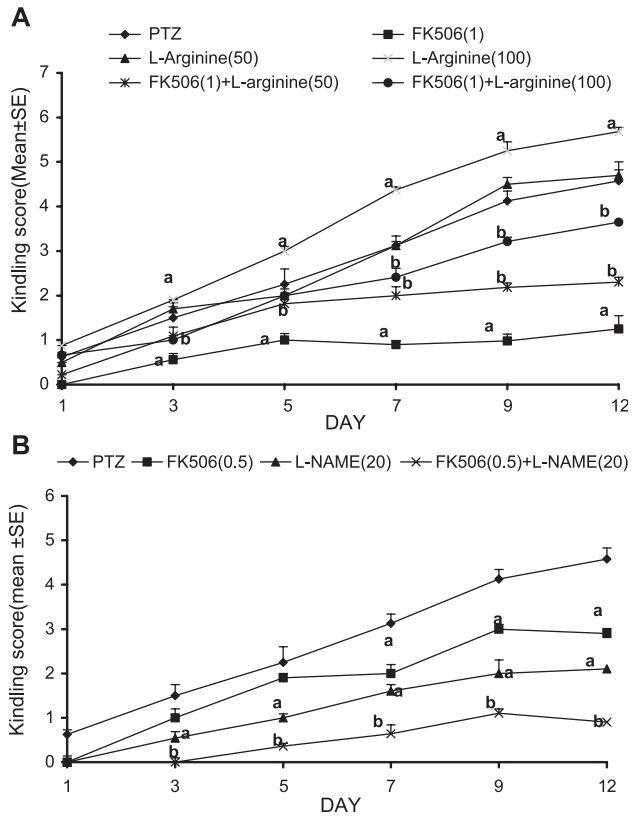

Fig. 3. Modification of effect of L-NAME (20 mg/kg ip) (A) and L-arginine (50–100 mg/kg ip) (B) by FK506 (0.5 or 1 mg/kg po) on PTZ-induced kindling (kindling score (mean ± SE), n=8 – 14). [a] $P < .05$ as compared with PTZ-treated group (ANOVA followed by Dunnett's test). [b] $P < .05$ as compared with FK506 (0.5 or 1 mg/kg)-treated group (ANOVA followed by Dunnett's test).

Table 1
Effect of FK506, L-NAME, arginine and their respective combinations on PTZ-mediated elevation on mice whole brain MDA levels

Treatment	Lipid peroxidation (nmol MDA/mg protein)
Control	0.89 ± 0.022
PTZ	3.458 ± 0.12[a]
FK506 (0.5)	2.31 ± 0.01[b]
FK506 (1)	1.1 ± 0.009[b]
L-Arginine (100)	5.98 ± 0.25[b]
L-NAME (10)	3.25 ± 0.14
L-NAME (20)	1.89 ± 0.088[b]
FK506 (1) + L-arginine (100)	3.446 ± 0.12[c]
FK506 (0.5) + L-NAME (20)	0.88 ± 0.1[d]
L-NAME (20) + L-arginine (100)	2.98 ± 0.18[e]

$n = 10-14$.

[a] $P < .05$ as compared with control (ANOVA followed by Dunnett's test).

[b] $P < .05$ as compared with PTZ-treated group (ANOVA followed by Dunnett's test).

[c] $P < .05$ as compared with FK506 (1 mg/kg).

[d] $P < .05$ as compared with FK506 (0.5 mg/kg)- and L-NAME (20 mg/kg)-treated group (ANOVA followed by Dunnett's test).

[e] $P < .05$ as compared with L-NAME (20 mg/kg).

3.3. Modification of effect of FK506 by L-arginine and L-NAME on PTZ-induced kindling

Co-administration of L-arginine (50–100 mg/kg ip) reversed the effect of protective effect of FK506 (1 mg/kg) as there was an increase in cumulative kindling score as compared to FK506 per se. On the other hand, L-NAME (20 mg/kg ip) potentiated the protective effect of FK506 (0.5 mg/kg) (Fig. 3).

3.4. Effect of FK506, L-NAME, L-arginine and their combinations on lipid peroxidation in PTZ-treated mice

Chronic treatment with PTZ induced oxidative stress as indicated by a significant raise in the whole brain MDA levels, compared with vehicle-treated group. FK506 and L-NAME dose dependently reversed the oxidative stress as indicated by a decrease in MDA level ($n = 10$, $P < .5$). On the other hand, L-arginine potentiated the PTZ-induced oxidative stress as indicated by elevated levels of MDA.

L-NAME (20 mg/kg) potentiated the effect of FK506 (0.5 mg/kg). L-Arginine (50–100 mg/kg), when given in combination with FK506 (1 mg/kg), reversed the protective effect of FK506 (1 mg/kg) as well as L-NAME as indicated by elevated levels of MDA as compared to L-NAME (20 mg/kg)- and FK506(1 mg/kg)-alone-treated mice (Table 1).

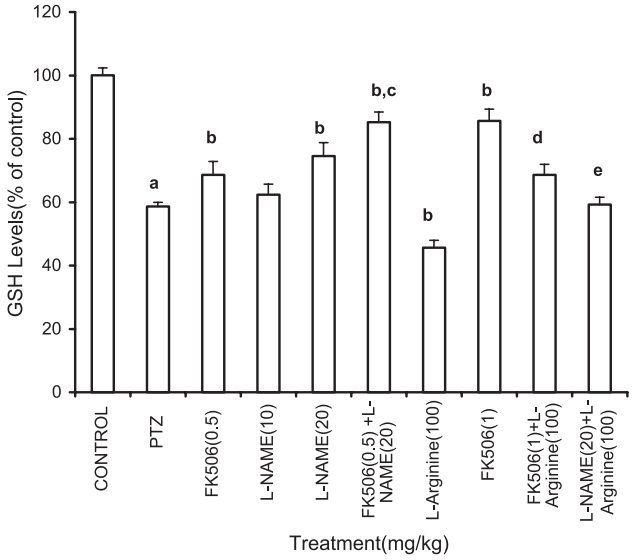

Fig. 4. Effect of FK506, L-NAME, arginine and their respective combinations on PTZ-mediated depletion of brain antioxidant enzyme SOD. Values expressed as percent response of vehicle-treated control group ($n = 8-10$). [a]$P < .05$ as compared with control (ANOVA followed by Dunnett's test). [b]$P < .05$ as compared with PTZ-treated group (ANOVA followed by Dunnett's test). [c]$P < .05$ as compared with FK506 (0.5 mg/kg)- and L-NAME (20 mg/kg)-treated group (ANOVA followed by Dunnett's test). [d]$P < .05$ as compared with FK506 (1 mg/kg). [e]$P < .05$ as compared with L-NAME (20 mg/kg).

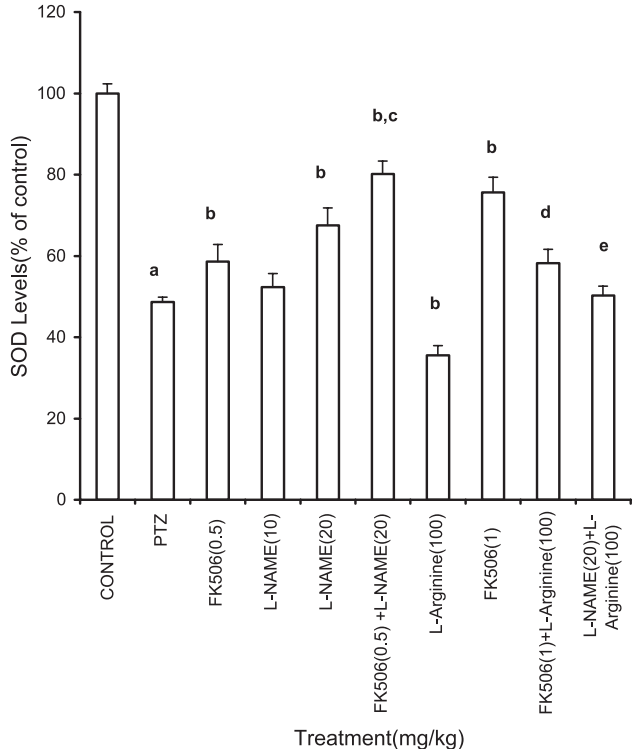

Fig. 5. Effect of FK506, L-NAME, arginine and their respective combinations on PTZ-mediated depletion in whole brain glutathione (GSH). Values expressed as percent response of vehicle-treated control group ($n = 8-10$).). [a]$P < .05$ as compared with control (ANOVA followed by Dunnett's test). [b]$P < .05$ as compared with PTZ-treated group (ANOVA followed by Dunnett's test). [c]$P < .05$ as compared with FK506(0.5 mg/kg)- and L-NAME (20 mg/kg)-treated group (ANOVA followed by Dunnett's test). [d]$P < .05$ as compared with FK506(1 mg/kg). [e]$P < .05$ as compared with L-NAME (20 mg/kg).

3.5. Effect of FK506, L-NAME, L-arginine and their combinations on whole brain enzyme levels in PTZ-treated mice

Chronic treatment of PTZ induced oxidative stress as indicated by a significant decrease in the whole brain GSH and SOD levels, compared with vehicle-treated group FK506, and L-NAME dose dependently reversed the oxidative stress as indicated by increase in GSH and SOD levels ($n=10$, $P<.5$). On the other hand, L-arginine potentiated the PTZ-induced oxidative stress as indicated by decreased levels of GSH and SOD levels as compared to PTZ-alone-treated mice.

L-NAME (20 mg/kg) potentiated the actions of FK506 (0.5 mg/kg). L-Arginine (50–100 mg/kg), when given in combination with FK506 (1 mg/kg), reversed the protective effect of FK506 (1 mg/kg) as well as L-NAME as there was a decrease in the GSH and SOD levels as compared to L-NAME- and FK506-alone-treated mice (Figs. 4 and 5).

3.6. Effect of FK506, L-NAME or L-arginine and their combinations on nitrite levels in PTZ-treated mice

Chronic treatment with PTZ induced an increase in NO levels as indicated by a significant raise in the whole brain nitrite levels, compared with vehicle-treated group. FK506 and L-NAME dose dependently reversed the increased nitrite levels ($n=10$, $P<.5$). On the other hand, L-arginine potentiated the PTZ-induced increased nitrite levels. L-NAME (20 mg/kg) potentiated the effect of FK506 (0.5 mg/kg). L-Arginine (50–100 mg/kg), when given in combination with FK506 (1 mg/kg), reversed the protective effect of FK506 (1 mg/kg) or L-NAME as indicated by elevated levels of nitrite as compared to L-NAME (20 mg/kg)- or FK506 (1 mg/kg)-alone-treated mice (Fig. 6).

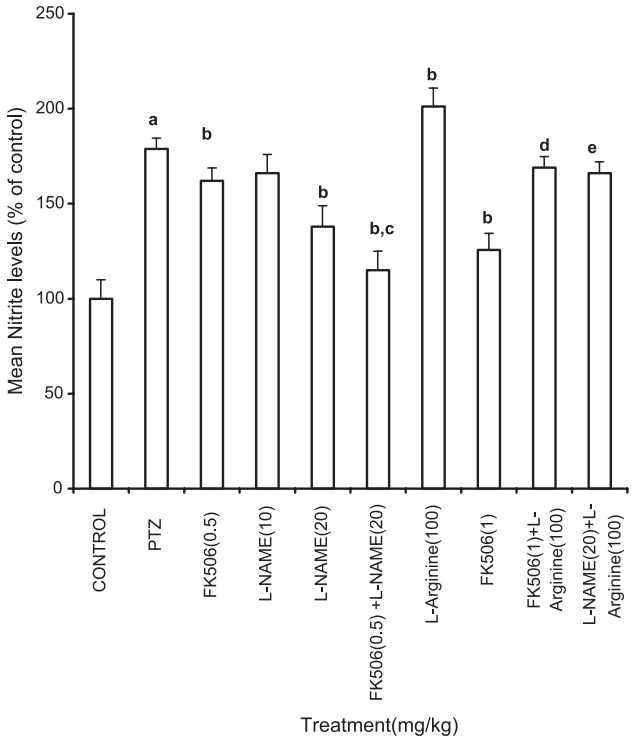

Fig. 6. Effect of FK506, L-NAME, arginine and their respective combinations on PTZ-mediated increase in nitrite levels. Values expressed as percent response of vehicle-treated control group ($n=8$ to 10). [a]$P<.05$ as compared with control (ANOVA followed by Dunnett's test). [b]$P<.05$ as compared with PTZ-treated group (ANOVA followed by Dunnett's test). [c]$P<.05$ as compared with FK506 (0.5 mg/kg)- and L-NAME (20 mg/kg)-treated group (ANOVA followed by Dunnett's test) [d]$P<.05$ as compared with FK506 (1 mg/kg). [e]$P<.05$ as compared with L-NAME (20 mg/kg).

4. Discussion

Kindling is a model of epilepsy and epileptogenesis. Kindling seizures in animals after daily electrical stimulation of the amygdaloidal complex through low intensity electric current have been described. The repeated administration of an initially subconvulsant dose of PTZ (a blocker of the Cl^- channel of $GABA_A$ receptors) (Corda et al., 1990) determines the appearance and progressive intensification of convulsant activity, culminating in a generalized seizure (i.e., chemical kindling) (Corda et al., 1991). PTZ-induced kindling is an experimental model of epilepsy that shares many features in common with electrical limbic kindling, but that primarily involves the neocortex (Barkai et al., 1994). Our results are in accordance with these findings.

One of the important candidates modulating cerebral circulation and brain damage during neonatal seizures is assumed to be NO (Takei et al., 1999b). NO is synthesized from L-arginine and oxygen by a calcium calmodulin-dependent NOS in a variety of cells, including endothelial cells, neurons and platelets (Moncada et al., 1991). Several evidences that demonstrate the excessive production of NO can be detrimental for the neuronal cells both in vitro and in vivo (Murphy, 1999). It has been reported that administration of 1,2,3,4-tetrahydro-9-amino-acridine (tacrine), a cholinesterase inhibitor in lithium chloride-pretreated rats, produces motor and electrocorticographic (ECoG) seizures and delayed hippocampal damage via mechanism that implicates abnormal production of NO. Under the above experimental conditions, systemic administration of L-NAME, a specific though not selective inhibitor of NOS (Moncada et al., 1991), abolishes in a stereoselective fashion the elevation of brain citrulline and prevents motor and ECoG seizures, and delayed hippocampal damage furthers implication excessive NO (Bagetta et al., 1995a,b). As observed in the present study, there was a potentiation of cumulative kindling score by L-arginine as assessed on 12th day of the study as compared to the control PTZ-treated mice. On the other hand, L-NAME decreased the cumulative kindling score.

There is a little evidence that free radicals are actively involved in physiological processes during oxidative stress induced by convulsants (Coyle, 1993; Halliwell et al.,

1991). Of all the free oxygen radicals that can occur in vivo, the hydroxyl-free radicals (OH) are considered to be most reactive and hazardous (Haliwell, 1992). There was a time course of whole brain OH formation after acutely induced PTZ seizures and in kindled rats following PTZ administration in comparison to the saline controls. When PTZ was administered, the content of dihydroxy butyric acid reflected the OH generation during the development of the convulsions, or after fully developed seizures (Christine et al., 1999), this demonstration of early seizure-induced OH formation fulfils the critical requirement in establishing the role of oxygen radicals in the pathophysiology of epilepsy.

Different mechanisms are conceivable for the increase of the free radicals in PTZ-induced convulsions. Previous studies have shown that both the administration of an acute seizure-inducing dose and the injection of seizure-inducing dose on kindled animals are associated with increase in the extra cellular glutamate levels. It may be assumed that further reason exist for the increased formation of OH^- in kindled animals during PTZ seizure, such as reduced activity of SOD, a major defense system for counteracting the toxic effects of reactive oxygen species such as O^{2-}. Despite enhanced activity, SOD-induced protection seems not to be sufficient to scavenge all O^{2-} generated during seizures because an intra-amygdala injection of SOD causes suppression of kindled seizures (Rocha et al., 1996; Bonfoco et al., 1995; Cassina and Radi, 1996).

Another source of OH radical is the peroxynitrate anion ($ONOO^-$), which is generated by the spontaneous reaction of O^{2-} and NO. When NO synthesis is enhanced, the formation of OH^- is favored strongly, thus initiating the processes of lipid peroxidation and formation of protein adducts, which induce cell damage. Moreover, excess NO may also promote an increase of O^{2-} production by binding to the heme moiety of the cytochrome *c* oxidases, the complex IV of the electron transport chain in the mitochondrial membrane. This binding may cause a transient inhibition of the electron flow yielding an increase in O^{2-} synthesis by complexes I and III (which are relatively insensitive to NO), thus favoring the intracellular production of peroxynitrate (Becker et al., 1995).

Following hippocampal kindling, an increase in NOS mRNA expression in CA1 and CA3 pyramidal layers and piriform cortex could be estimated 12–24 h after hippocampal stimulation demonstrated the L-NAME; an inhibitor of NOS was able to suppress the PTZ kindling development significantly (Elmer et al., 1996), further suggesting the role of NO in PTZ-induced kindling.

In our study, there was an increase in MDA levels (indicator of lipid peroxidation and thus formation of free radicals, supported by enzyme assays) by PTZ administration, since L-arginine potentiated the MDA levels and L-NAME restored the MDA levels.

In our study, FK506 not only decreased the mean kindling score but also reversed the effect of L-arginine on PTZ-induced kindling. FK506 also potentiated the protective effect of L-NAME on PTZ-induced convulsions when it was combined with FK506.

FK506, an immunosuppressant drug, binds to FK506-binding protein (FKBP). One target of the FK506/FKBP complex is the calcium/calmodulin-dependent protein phosphatase calcineurin, whose activity is inhibited upon interaction with FK506/FKBP (Gold et al., 1995). FK506 treatment increases phosphorylation level of calcineurin substrates including NOS. As a potent neuroprotective agent in vitro and in vivo, FK506 increases NOS phosphorylation and decreases NO production (Zhang and Steiner, 1995). Thus, the neuroprotective effect of FK506 may be attributed to inhibition of NOS activity (Macleod and Butcher, 2002). Recently, it has been reported an independent mechanism for the antioxidant properties of FK506, and it was shown that the antioxidant properties of immunophilin ligands are independent on the FKBP12 pathway (Tanaka et al., 2002). Thus, administration of FK506 in PTZ might have decreased the formation of free radicals per se or through the inhibition of NOS, and thus decreased the NO formation. This observation is well supported by biochemical analysis in which FK506 decreased the MDA levels as compared to the PTZ-treated mice. FK506 inhibited the elevated NO levels due to L-arginine treatment, due to its property of NOS inhibition, and thus reversed the detritus effects of L-arginine on PTZ-induced kindling. Potentiation of protective effect of FK506 by L-NAME and reversal of protective effect of FK506 by L-arginine support our hypothesis. Reversal of L-NAME protective effect in PTZ-induced kindling by L-arginine confirms this.

Although Homayoun et al. (2002) have shown the effectiveness of cyclosporine in PTZ-induced kindling, cyclosporin A is known to cause renal failure on chronic treatment with the dose they have employed (Satyanarayana and Chopra, 2002). FK506 is relatively a safer drug, and the dose employed in the present study (1 mg/kg) has been reported to be insufficient to prevent rejection of neuronal transplants in the rat (Sakai et al., 1991) (lacks immunosuppressive effects). Further lower dose, that is, 1% of the immunosuppressive dose, has shown neuroprotection in animal models nerve injury or ischemia (Kihara et al., 2001).

FK506 is reported to be nearly 200 times more potent than cyclosporine in producing neuroprotection and it has better penetration across blood brain barrier as compared to cyclosporine. Therefore, it is preferred as a neuroprotective agent especially in the cases of cerebral ischemia (Butcher et al., 1997).

There are controversial reports of effectiveness of FK506 in kindling epilepsy as Suzuki et al. (2001) have shown proconvulsant effect of FK506, but Homayoun et al. (2002) reported the effectiveness of neuroimmunophilin ligands in kindling epilepsy and the effectiveness of neuroimmunophilin ligands in kindled rats (Moia et al., 1994). Therefore, FK506 is expected to show beneficial effects in kindling

epilepsy. The report by Suzuki et al. does not clarify the exact mechanism of the actions of FK506, but our study shows the effectiveness of FK506 in kindling epilepsy, either due to the antioxidant mechanism of FK506 and/or by indirect NOS inhibition.

Further, to confirm the second hypothesis, we measured nitrite levels in the brain. Results clearly indicate that the neuroprotective effect of FK506 is through the NOS inhibition as pretreatment of FK506 considerably reversed the increased nitrite levels caused by PTZ-induced kindling. The protective effect of FK506 was reversed by L-arginine and potentiated by L-NAME, an NO donor and an NOS inhibitor, respectively.

Thus, in conclusion, FK506 when chronically given in PTZ-treated mice dose dependently showed the protective effect. It not only reversed the deleterious effect of L-arginine but also potentiated the protective effect of L-NAME on PTZ-induced kindling. The biochemical observations not only clearly implicated the role of free radicals in PTZ-induced kindling but also explained the possible mechanism of protective effect of FK506, through the reduced formation of free radicals either directly or indirectly by NOS inhibition, thereby reducing NO formation.

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