National Journal of Physiology, Pharmacy and Pharmacology

RESEARCH ARTICLE

# Fructose-1,6-diphosphate and vitamin C combination increases seizure threshold in chemical kindling models

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**Received:** September 15, 2016; **Accepted:** September 30, 2016

## ABSTRACT

**Background:** Fructose-1,6-diphosphate (FDP), through its activity in glucose metabolism, has anticonvulsant efficacy whereas vitamin C acts as neuromodulator. **Aims and Objectives:** To assess the antiepileptic effects of FDP, vitamin C and their combination in intravenous pentylenetetrazole (PTZ) and intracranial carbachol kindling model. **Materials and Methods:** PTZ kindling was induced by intravenous infusion of PTZ (10 mg/ml; infusion rate: 1 ml/min) to rats. For carbachol kindling, intracranial administration of carbachol (1 µl; 5 µg/ml) was done at well-spaced intervals by introducing maximum three injections a day, and maximum nine administrations until stage 5 epileptic convulsions were produced. The treated group animals received FDP, vitamin C or both, 28 days before the kindling. During kindling, time latency to reach a seizure stage, electroencephalogram (EEG) recording, and frequency of stimulation were recorded. Brain homogenate parameters included measurement of levels of neurotransmitters, glutathione (GSH), and malondialdehyde (MDA). One-way ANOVA was used for determination of a significant difference between the groups. **Results:** Slowing of seizure progression, increase in seizure threshold and required number of stimuli to reach to stage 5 convulsion was highest with FDP and Vitamin C combination followed by FDP and Vitamin C alone treatment. EEG data during the PTZ infusion indicated ictal epileptiform in disease control group and not in treatment groups. The test drugs either alone or in combination did not alter glutamate and aspartate levels in brain but increased the γ-aminobutyric acid and GSH whereas reduced MDA levels. **Conclusion:** It can be concluded that both FDP and Vitamin C has beneficial effects in chemical induced kindling and their combination shows synergistic effects.

**KEY WORDS:** Chemical Kindling; Pentylenetetrazole; Carbachol; Electroencephalogram

## INTRODUCTION

Despite the current optimal drug therapy, there are 50 million people living with epilepsy worldwide, from which 30% patients may develop refractory epilepsies or need combinations.[1,2] The current antiepileptic medication has many side effects and adverse drug reactions.[3] Thus, new drugs with minimal side effects and effective in refractory epilepsy are needed.

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| **DOI:** 10.5455/njppp.2017.7.0927330092016 | |

Studies have shown that ketogenic diet (KD) - a high-fat, low carbohydrate, adequate protein diet - can be used to manage refractory epilepsy in children, by forcing the body to use fat instead of carbohydrates[4] and a decrease in glycolysis has been suggested to be the mechanism of this diet. Taking this into account, a cardioprotective, fructose-1,6-diphosphate (FDP) was selected. It has been shown to regulate glucose utilization and enhance flux of glucose into the pentose phosphate pathway and preserve cellular glutathione (GSH) levels.[5] FDP decreases, the expression of brain-derived

neurotrophic factor (BDNF) and its receptor tropomyosin-related kinase (TrkB) which are found to be essential for seizure activity.[6] It also acts on the $Na^+$-$K^+$-$Cl^-$ cotransporter (KCC1) and $K^+$-$Cl^-$ cotransporter (KCC2), the levels of which are imbalanced in epilepsy both the cotransporters manage intracellular chloride concentrations and affects γ-aminobutyric acid [GABA] induced neuronal currents).[7]

Vitamin C plays neuroprotective function by inhibiting oxidative stress by blocking the efflux, rather than influx, of calcium, and therefore, it can interfere with the mechanisms of neurotransmitter release and/or uptake from neuronal terminals. It also decreases peroxidation of lipids and works as a neuromodulator.[8]

In this study, both FDP and vitamin C were used alone and in combination, followed by chemical kindling using two different chemicals: Pentylenetetrazole (PTZ) intravenously and carbachol intracranially. The anticonvulsant activity of FDP and vitamin C combination was evaluated.

## MATERIALS AND METHODS

The experimental protocol of this study was approved by local Institutional Animal Ethics Committee. Female Wistar rats of 4-8 weeks, weighing 200-300 g were selected and housed at 25 ± 1°C and 70% relative humidity under a 12 h light/12 h dark cycle with free access to water and conventional laboratory diet. All the animals were divided in four groups: Disease control, FDP treated (150 mg/ml, p.o.), vitamin C treated (500 mg/kg, i.p.), and FDP (150 mg/ml, p.o.) + vitamin C (500 mg/kg, i.p.) treated. After the treatment for 28 days, PTZ infusion test was performed.

### PTZ Infusion Test

PTZ solution (10 mg/ml) was filled in the rate controlled infusion set, and the infusion rate was set as 1 ml/min.[9] Animals were restrained such that the limbs and head were freely moving. Electroencephalogram (EEG) recording was performed before and during the infusion, using student's physiograph (Bio-devices, Ambala). EEG electrodes were inserted in three different regions of the scalp (one electrode in frontal region, and two in parietal region). Conducting gel was applied on the electrode surface. One electrode was used as ground electrode. The recording was performed twice; before the infusion, for the normal state EEG (which was considered as baseline EEG) and the during the infusion test.

After recording the baseline EEG, a butterfly cannula (needle size 24-gauge) was attached to the prefilled infusion set. The needle of the cannula was inserted into the tail vein. During the infusion, EEG pattern was recorded. Infusion was continued until the rat exhibited its first overt bilateral myoclonic twitch of the forelimbs.[10] Seizure severity was recorded as seizure stage as shown in Table 1.

The treatment was then discontinued. After 10 days, stereotaxic surgery was performed whereby a cannula was implanted in the animals by surgical procedure, which was followed by carbachol kindling.

### Carbachol Kindling Model

The animals were stereotaxically implanted with permanent, stainless steel guide cannulae (0.4 mm o.d.) under ketamine (40 mg/kg; i.p.), and diazepam (6 mg/kg; i.p.) anesthesia.[11,12] The cannulae were implanted in amygdala at coordinates anterior-posterior = 4.8 mm, dorsal-ventral = 3 mm, and medial-lateral = 5 mm using stereotaxic apparatus (Stoelting Co., USA).[13] Guide cannulae were fixed to the skull using retaining screws and rapid-setting dental acrylic cement.[14] One week recovery postoperatively was given to the rats before the start of treatment. After the treatment of 28 days (as in the previous model), carbachol kindling was carried out.

Carbachol (1 μl) was injected thrice a day, at an interval of 3 h, until the animals were observed for stage 5 seizures. Following each infusion, animals were observed for a minimum of 30 min, during which seizure stage were noted. The seizure stages were assigned as shown in Table 1.[15]

After the last stimulation, within 1 h, the animals were sacrificed and brains were isolated. Brain homogenate was used for the measurement of glutamate, aspartate, GABA, malondialdehyde (MDA), and reduced GSH.

### Aspartate and Glutamate Estimation

Aspartate and glutamate estimation was carried out by high performance thin layer chromatography technique using brain tissue samples.[16,17]

### GABA Estimation

GABA concentration determination was based on a fluorometric assay that depends on the formation of a fluorescent product from the reaction between GABA and ninhydrin at alkaline pH and in the presence of glutamate.[18] After analyzing the test samples, the remaining homogenates were mixed and then equal volume of homogenate mixture was added to different standard concentrations. Same procedure was performed as the test and absorbance was measured. The test concentration was determined from the calibration curve.

### MDA Estimation

MDA was determined by reaction with thiobarbituric acid (TBA) under high temperature (90-100°C) and acidic

**Table 1:** Seizure stages in PTZ and carbachol induced kindling model

| Seizure stage | PTZ model[8] | Carbachol model[13] |
|---|---|---|
| Stage 1 | Vibrissal/jaw twitching | Repeatable vibrissae twitches associated with or followed by increased perioral movements, jaw movements and/or licking and swallowing |
| Stage 2 | Myoclonic jerking with vocalization | Repetitive eye blinking, ear twitching and/or head jerks |
| Stage 3 | Repeated forelimb clonus | Rearing and clonic–tonic movements of the front legs with maintenance of body balance |
| Stage 4 | Jerking of whole body | Trunk muscles with transient loss of balance usually seen during rearing |
| Stage 5 | Forelimb/hindlimb TE | Generalized tonic–clonic convulsions with loss of balance, cyanosis and accompanying autonomic symptoms such as heavy respiration, salivation, urination, and defecation |

TE: Tonic extensor, PTZ: Pentylenetetrazole

**Table 2:** Effect of drug treatments on neurotransmitters, MDA and GSH

| Groups | Brain homogenate parameters | | | | |
|---|---|---|---|---|---|
| | Aspartate (µmol/g) | Glutamate (µmol/g) | GABA (µmol/g) | MDA (nM/mg of protein) | GSH (µmol/g of wet tissue) |
| Control | 2.57±0.27 | 15.30±1.45 | 0.62±0.06 | 106.74±6.07 | 0.43±0.09 |
| FDP | 2.26±0.23 | 12.62±1.34 | 1.15±0.30 | 101.01±8.27 | 1.02±0.14* |
| Vitamin C | 2.32±0.09 | 13.19±0.66 | 0.81±0.15 | 69.16±2.85‡\|\| | 1.21±0.11† |
| FDP+vitamin C | 2.21±0.24 | 12.17±0.93 | 1.47±0.05* | 73.11±8.41†§ | 1.78±0.15‡§** |

Significantly different from disease control group (*$P$<0.05, †$P$<0.01, ‡$P$<0.001), significantly different from FDP treated group (§$P$<0.05, ||$P$<0.01), significantly different from vitamin C treated group (**$P$<0.05). Values were expressed in mean±SEM ($n$=6). SEM: Standard error of the mean, MDA: Malondialdehyde, GSH: Glutathione, GABA: γ-Aminobutyric acid

condition to yield a pink MDA-TBA adduct which was measured by ultraviolet spectrophotometry using wavelength 532 nm.[19]

### GSH Estimation

This estimation was performed using Ellman's Reagent 5,5'-dithiobis-(2-nitrobenzoic acid) which reacts with GSH to yield a mixed disulfide and 2-nitro-5-thiobenzoic acid or TNB (yellow-colored product), measured colorimetrically at 412 nm. The activity was expressed as µmol/g brain tissue.[20]

### Statistical Analysis

Data were presented as mean ± standard error of the mean (SEM). Statistical evaluation of mean seizure stage in PTZ kindling was analyzed using one-way analysis of variance (ANOVA) followed by Dunn's multiple comparison test. Total seizure stage was analyzed using two-way ANOVA. Other tests were performed using one-way ANOVA followed by Tukey's multiple comparison test. All the tests were performed using GraphPad Prism® version 5.01.

## RESULTS

### PTZ-Induced Kindling Parameters

#### *EEG recordings*

The disease control group showed increased amplitude and decreased frequency, in contrast to treatment groups, where an increase in amplitude was not observed (Figure 1).

#### *Time latency and mean seizure stage*

Time latency to reach each seizure stage was recorded during PTZ infusion and graphically represented as time latency versus seizure stage. As shown in Figure 2a, the treatment groups showed a significant increase in time latency as compared to disease control group. A gradual increase in time latency of treated groups was observed as the seizure stage progressed.

In Figure 2b, the control group animals reached stage 5 seizure whereas mean seizure stage of FDP, vitamin C and combination treated groups were 4.16 ± 0.47, 4.33 ± 0.49 and 3.6 ± 0.33, respectively, depicting a significant difference between combination treated and disease control group.

### Carbachol-Induced Kindling Parameters

A total number of stimulations (mean) required by FDP + vitamin C treated group animals were 7.16 ± 0.71 which was higher than disease control (3.33 ± 0.71), FDP treated (6 ± 1.21), and vitamin C treated (4 ± 0.73) as shown in Figure 3a. Similarly, time latency to reach stage 5 seizure was highest in combination group with 43.33 ± 5.26 min when compared to other groups (Figure 3b). The least percentage protection value was of disease control animals followed by vitamin C and then FDP. Combination treatment showed the highest protection of 79.62%± 5.30%.

### Brain Homogenate Parameters

The results of brain homogenate parameters viz. aspartate, glutamate, GABA, GSH and MDA are represented in Table 2.

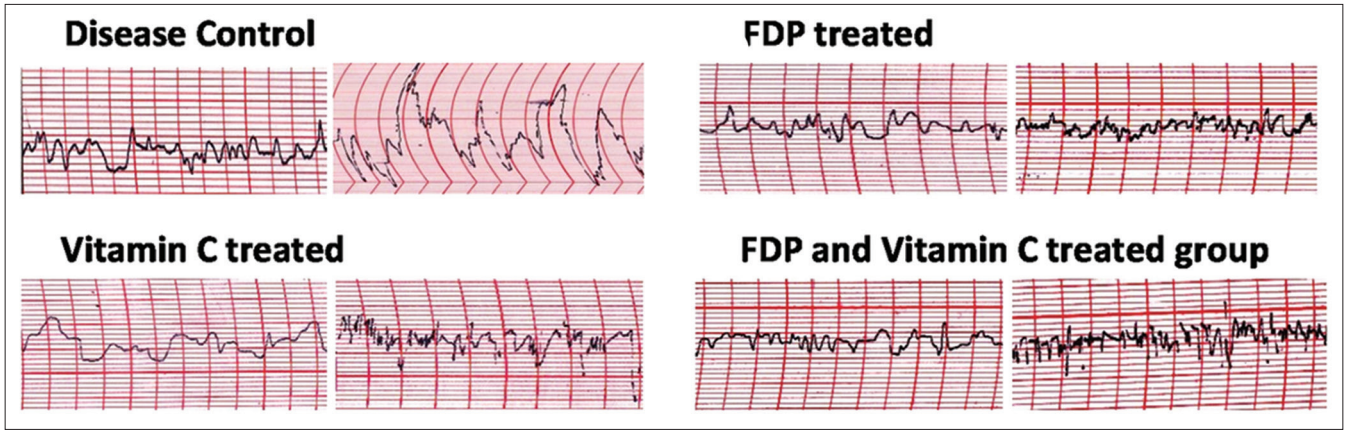


**Figure 1:** Baseline electroencephalogram (EEG) and EEG recordings during infusion of animals of disease control, fructose-1,6-diphosphate (FDP) treated, vitamin C treated and FDP and vitamin C treated group, respectively

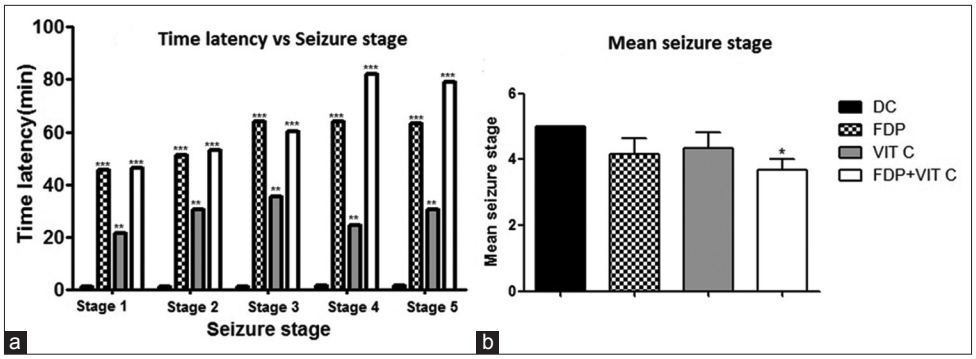


**Figure 2:** (a) Graph of time latency versus seizure stage in pentylenetetrazole (PTZ) kindling model. (b) Graph of mean seizure stage observed in different groups in PTZ kindling model. Values are expressed as mean ± standard error of the mean ($n$ = 6) (significantly different from disease control group [*$P$ < 0.05, **$P$ < 0.001, ***$P$ < 0.001])

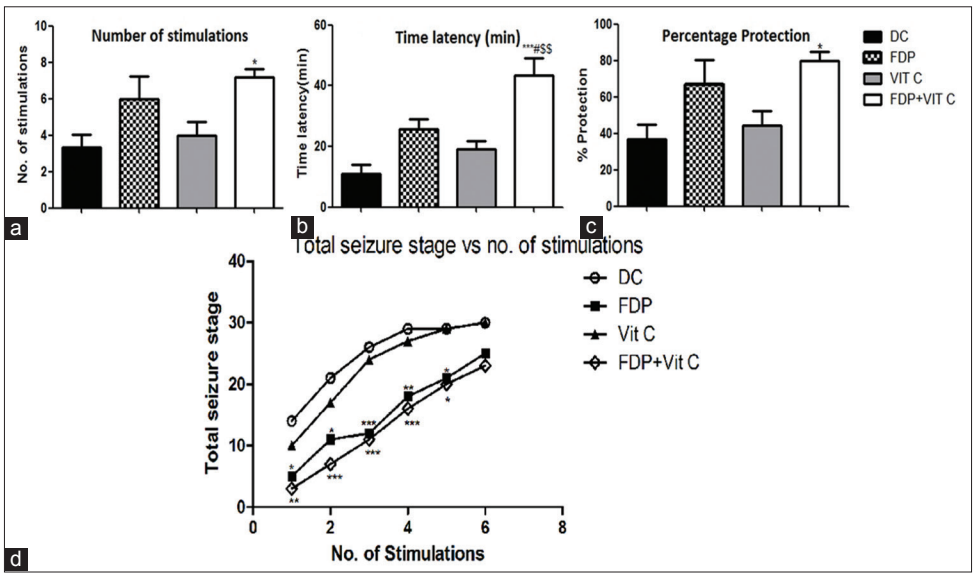


**Figure 3:** (a) Number of stimulations required in carbachol kindling model. (b) Time latency to reach stage 5 in carbachol kindling model. (c) Percentage protection for carbachol kindling model. (d) Total seizure stage versus number of stimulations in carbachol kindling. Values are expressed as mean ± standard error of the mean ($n$ = 6) (significantly different from disease control group (*$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001); significantly different from FDP treated group ($^{\#}P$ < 0.05), significantly different from vitamin C treated group ($^{\$\$}P$ < 0.01)

## DISCUSSION

Changes in glucose metabolism alter the seizure susceptibility and are evident in FDP, KD, and 2-deoxyglucose (2DG), a compound acting on glucose uptake. FDP was found to be more effective than KD and 2DG against seizures induced by pilocarpine, kainic acid, and PTZ.[5] Moreover, literature suggests impaired cognitive function due to KD and inhibition

of memory consolidation by subcutaneous administration of 2DG to a chick.[5] On the other hand, FDP allows glucose utilization in specific embodiments which results in less cognitive impairment making it suitable for clinical use as an anticonvulsant. For this study, dose selection of FDP was done based on the study by Lian et al., which demonstrated oral administration of FDP and its anticonvulsant effects[21] whereas vitamin C showed its effects at 500 mg/kg in PTZ and pilocarpine-induced models,[2,8] which eliminates the need for dose optimization.

The need for PTZ intravenous infusion arose as it is found to be better than i.p. or s.c. administration and can separately determine thresholds for clonic–tonic seizures in the same animals and evaluate effects of separate drugs on different seizure types.[22] In PTZ model, the EEG recordings of the disease control animals represented ictal epileptiform progressing to an epileptiform burst pattern, accompanying clonic activity. In contrast to this, treatment groups do not show such activity. The data suggest that FDP and Vitamin C combination group significantly delayed the acquisition of seizures induced by PTZ kindling as the time latency of combination group to reach each stage was increased significantly.

The seizure acquisition in amygdala-kindled animals is faster than hippocampus and caudate nucleus due to which amygdala injection was preferred.[13] In the carbachol kindling, the rats treated with FDP and Vitamin C combination required higher numbers of stimuli than the disease control and single drug treated groups to develop stage 5 seizures (i.e. generalized tonic–clonic seizures) and the kindling progression of the combination group was slow as compared to the disease control group. Therefore, the combination seems to be more effective in increasing the seizure threshold than the individual drug treatment in both the kindling models.

The reported normal brain glutamate and aspartate levels in rat were 9.26 ± 0.33 and 1.93 ± 0.22 μmole/g brain wet weight, respectively.[23] The glutamate and aspartate levels in the brain homogenate of all the groups were higher than the reported levels and showed no significant difference from the disease control group, suggesting a probable increase in levels due to kindling. This may be due to the cholinergic stimulation, which led to an imbalance between excitatory and inhibitory pathway and increase in glutamate levels which ultimately activated N-methyl-D-aspartate.[13,24,25]

The normal GABA level in rat brain homogenate was reported as 1.49 ± 0.06 μmole/g brain wet weight.[23] Brain homogenate of kindled animals showed GABA levels lower than this level, and this effect can be attributed to kindling, in which there is selective lesion of GABA synthetic pathway.[24] Higher MDA levels in the disease control group than treated groups indicate that the existing lipid peroxidation could be responsible for neuronal damage which led to decreased seizure threshold and the antioxidant capacity was not enough to protect brain cells against oxidative damage.[8] The data from Vitamin C treated groups suggest a positive reduction in the lipid peroxidation levels as compared to disease control group, which indicates its neuroprotective actions.

Reduced GSH levels are an important indicator of mitochondrial and cellular health, and mitochondrial dysfunction have been implicated in seizures and epilepsy.[26] FDP preserves, the GSH levels in the cells and Vitamin C helps to restore GSH. Thus, the observations of combination group show a synergistic effect. All these effects may be contributing in decreasing the excitation and delaying seizure acquisition in the combination group.

Various mechanisms for FDP's efficacy in epilepsy has been proposed *viz*. preservation of cellular GSH by enhancing flux of glucose in pentose phosphate pathway, decreasing expression of BDNF and its receptor TrkB and managing intracellular chloride channel concentrations by acting on various transporters. The effectiveness of FDP in the models tested may be due to its action on a final common pathway in epileptogenesis that is independent of the mechanism of seizure initiation.[5] FDP illuminates novel approaches for controlling seizures and further investigations may lead to the development of a small molecule replacement for the complex KD treatment.[7] Vitamin C can exert neuroprotective function during acute phase of seizures.[27] GABA inhibition (in PTZ model) and the muscarinic receptor activation (in carbachol model) seem to be involved in the genesis of seizures and causes brain oxidative stress. Vitamin C consolidates cell membrane, supports superoxide dismutase and catalase activity, playing a defensive role. Hence, inhibition of free radical production by vitamin C is promising as supplementary therapy of epilepsy.[28]

## CONCLUSION

In conclusion, the combination of FDP and vitamin C, exhibiting neuronal protection and reduction in neuronal excitability, seems to be effective in the treatment of generalized epilepsy.

## ACKNOWLEDGMENTS

This project was supported by Dr. Mukul Jain, Senior Vice President, Zydus Research Centre by providing Stereotaxic instrument to our institute and Dr. Amit Johrapurkar, with his team in guiding the surgical procedures.

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**How to cite this article:** Samariya BP, Shah GB. Fructose-1,6-diphosphate and vitamin C combination increases seizure threshold in chemical kindling models. Natl J Physiol Pharm Pharmacol 2017;7(3):259-264.

**Source of Support:** Nil, **Conflict of Interest:** None declared.