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Original Research

Thymoquinone alone or in combination with phenobarbital reduces the seizure score and the oxidative burden in pentylenetetrazole-kindled rats

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Key Words
Pentylenetetrazole; Phenobarbital; Rats; Thymoquinone

Abstract
Epilepsy is one of the most common neurological diseases that effect many functions of the brain in a pathologically disturbed manner. Treatment goals include the control of the frequency of seizures with absence of side effects. Phenobarbital (PB) is the drug of choice for treatment of many kinds of epilepsy. Since oxidative stress has been implicated in the pathophysiology of epilepsy, the present study was designed to assess the anticonvulsant potential of thymoquinone given alone or in combination with PB in pentylenetetrazole (PTZ)-kindled rats and to determine if thymoquinone can protect against oxidative burden in the current rat model. The results showed that the combination of PB and thymoquinone had additive anticonvulsant effect compared to monotherapy with PB. In addition, although treatment with PB alone showed a significant improvement in plasma malondialdehyde (MDA), erythrocyte reduced glutathione (GSH) levels, and erythrocyte glutathione peroxidase (GPx) activity than PTZ-kindled rats, combination with thymoquinone showed more significant improvement in plasma MDA, erythrocyte GSH levels, erythrocyte GPx and glutathione reductase (GRd) activities compared to PTZ-kindled rats or rats received single treatment with PB. These results provide evidence that thymoquinone may have a significant anticonvulsant and antioxidant effect in the current model of chronic epilepsy when combined with PB.

INTRODUCTION

Epilepsy is one of the most common neurologic diseases of the brain, affecting at least 50 million people worldwide [1]. It is the most common serious primary disease of the brain, accounting for 1% of the global burden of disease [2] and knows no age, racial, social class, geographic, or national boundaries [3]. Epilepsy denotes any disorder characterized by recurrent seizures due to abnormal paroxysmal neuronal discharge in the brain. Symptoms range from sensory absences to convulsive movements and loss of consciousness [4].

The goals of treatment of epilepsy include the control of the frequency of seizures, allowing the patient to live an essentially normal life, and the absence of side effects or drug-drug interactions. Because therapy is extended for many years, often a life time, chronic side effects must be considered [5;6]. Phenobarbital is the drug of choice for neonatal seizures. It is also useful in generalized seizures, mixed seizures, tonic-clonic seizures, and may be useful in patients with partial seizures [7]. This agent is CNS depressant. It elevates seizures threshold by decreasing postsynaptic excitation, possibly by stimulating postsynaptic GABAergic inhibition responses [8]. Also, phenobarbital is a potent enzyme inducer that increase the elimination of any drug metabolized by phase I oxidative process [9].

Oxidative stress has been implicated in the pathophysiology of neurologic conditions including epilepsy, Alzheimer's disease, Parkinson's disease and Huntington's disease [10, 11]. Oxidative stress is a disparity between the rates of free radical production and elimination. This imbalance is initiated by

numerous factors that may accelerate neuronal degeneration and atherosclerosis in epileptics [10, 12-14].

Nigella sativa is a black seed that is believed to be indigenous to the Mediterranean region. Recently, the plant has been subjected to a range of pharmacological investigations justifying its broad traditional therapeutic value [15]. Nowadays, there is an increased demand for using plants in therapy "back to nature" instead of using synthetic drugs which may have adverse effects that may be more dangerous than the disease itself [16]. Many effects have been described for the seeds of *Nigella sativa* and their constituents including its antioxidant role [17, 18]. *Nigella sativa* has been recently known for its anticonvulsant effect; in particular its main constituent, thymoquinone, has been reported to acquire anticonvulsant activity in mice [15, 19]. Therefore, this study was designed to assess the anticonvulsant potential of thymoquinone given alone or in combination with phenobarbital (PB) against pentylenetetrazole (PTZ)-induced kindling in rats, and to determine if thymoquinone could be used for its antioxidant properties to reduce the oxidative burden that might enhance the response to the anticonvulsant agent, PB.

MATERIALS AND METHODS

Animals

Thirty male albino rats were used in this study. Animals had an initial body weight ranging from 100 to 130 g. They were kept under controlled laboratory conditions, normal light-dark cycle, temperature 25-30°C, and relative humidity of 55-65%. The rats were housed in stainless steel cages, with free access to food and water, in groups of two. All the experiments were performed between 10:00 a.m. and 2:00 p.m. to minimize circadian influences on seizure susceptibility.

Drugs and chemicals

Pentylenetetrazole (PTZ) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in sterile saline. Phenobarbital sodium was kindly provided by Alexandria Pharmaceutical Industrial Company (Alexandria, Egypt). Thymoquinone was also purchased from Sigma-Aldrich and was dissolved in olive oil. All other chemical were analytical grade or obtained from Sigma-Aldrich.

Experimental design

As shown below animals were randomly divided into 5 groups; each has 6 rats:

-*Group I (control group):* rats received 10 injections of normal saline parallel to each PTZ injection.

-*Group II (kindled group):* Rats were injected with subconvulsive doses of PTZ (35 mg/kg/48 h, i.p.), for a total of 10 injections [20].

-*Group III (kindled rats pretreated with PB):* rats were pretreated with PB (30 mg/kg, i.p) 60 min before each PTZ injection (35 mg/kg/48 h, i.p.)[20].

-*Group IV (kindled rats pretreated with thymoquinone):* rats were pretreated with thymoquinone (20 mg/kg, p.o.) 60 min before each PTZ injection (35 mg/kg/48 h, i.p.)[21]

-*Group V (kindled rats pretreated with PB and thymoquinone):* Rats were pretreated with PB (30 mg/kg, i.p) in addition to thymoquinone (20 mg/kg, p.o.), 60 min before each PTZ injection (35 mg/kg/48 h, i.p.). Thymoquinone was administered by oral gavage before each injection of PTZ.

Kindling procedure and scoring

Rats were randomly allocated to kindled and control (saline injected) groups. Rats were injected with subconvulsive doses of PTZ (35 mg/kg, i.p; every 48 h) for a total of 10 injections. After each PTZ injection, the convulsive behavior was observed for 30 min and classified according to Racine rating scale [21], as shown in Table 1. The average score was calculated for each group at the end of the experiment.

Blood collection

Changes that are associated with the kindling process would be expected to be long-lasting, accordingly, in the present study blood collection was performed within 5 h after the final PTZ injection. After the last PTZ injection, all rats were sufficiently anaesthetized with isoflurane. Then, blood samples were obtained from the orbital sinus with a microhematocrit blood tube [22]. Blood was collected on EDTA and centrifuged at 4000 rpm and at temperature of 4°C for 10 min to obtain the plasma (supernatant) and erythrocytes (pellet)[23] .

Table 1. Racine [21] rating scale for evaluation of seizures

Symptoms	Score
No seizure response	0
Immobility, eye closure, ear twitching, sniffing, facial clonus	1
Head nodding associated with more severe facial clonus	2
Clonus of one forelimb	3
Bilateral forelimb clonus without rearing	3.5
Bilateral forelimb clonus with rearing	4
Falling on a side (without rearing), loss of righting reflex accompanied by generalized tonic-clonic seizures	4.5
Rearing and falling on back accompanied by generalized tonic-clonic seizures	5

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Determination of plasma malondialdehyde

Plasma was analyzed for MDA by using the method of Yagi [24] that is based on the reaction with thiobarbituric acid to yield a colored product. The optical density of the reaction product was measured at 532 nm using 1,1,3,3-tetramethoxypropane as a standard.

Assay for reduced glutathione and antioxidant enzymes activities

Erythrocytes were washed with isotonic solution of sodium chloride and centrifuged at 3000 rpm for 10 min where erythrocyte pellets were separated and resuspended in cold distilled water to obtain the erythrocyte lysate [23, 25]**.** The resulting erythrocyte lysate was used for measurement of glutathione (GSH) homeostasis [26, 27]**,** determination of glutathione peroxidase (GPx)[28] and glutathione reductase (GRd) activities [29]**.** In addition, plasma protein concentration was measured as described by Lowry *et al* [30].

Statistics

Results were collected and expressed as mean ± SEM. For multiple comparisons of behavioral ratings, Quantitative data were compared using a repeated measures one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. Statistical significance was set at $P < 0.05$. Results were analyzed by using the Statistical Package for the Social Sciences software, version 17 (SPSS Inc., Chicago, IL, USA).

RESULTS

In the current study, there was an increase in severity of seizure in rats with repeated administration of subcovulsive doses of PTZ. Treatment with PB, thymoquinone, or the combination of PB and thymoquinone caused significant depression in the seizures exhibited by the rats. Meanwhile, the decrease in seizure resulted from addition of thymoquinone to PB was insignificant in comparison with the result obtained by using PB alone (Fig.1).

Regarding the biochemical results, Fig.2 shows significant increase in the plasma LP levels in rats kindled with PTZ than the control group. However, treatment with PB, thymoquinone, or the combination of PB and thymoquinone has significantly decreased the plasma LP levels if compared to the rats injected by PTZ alone. Meanwhile, further significant decrease in the plasma LP levels was observed after addition of thymoquinone to PB comparing to rats that treated with PB alone.

The erythrocyte biochemical analysis showed insignificant changes in the total GSH levels in all groups as shown in Fig.3. While Fig.4 shows

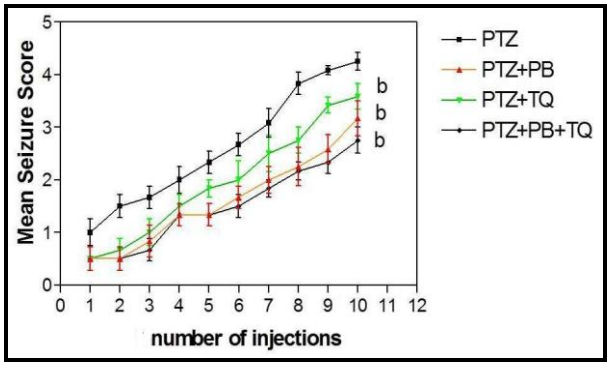

Figure 1. Mean seizure score exhibited by the rats after each injection of PTZ and effect of treatment with phenobarbital (PB), thymoquinone (TQ) and their combination.

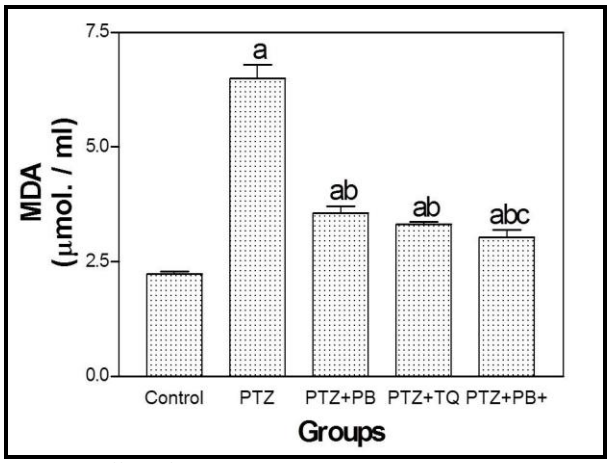

Figure 2. Effect of phenobarbital (PB), thymoquinone (TQ) or their combination on serum MDA level in PTZ-kindled rats. Results are expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post-hoc test at $P < 0.05$. [a]Significantly different from the control group, [b]significantly different from PTZ-kindled rats, and [c]significantly different from PTZ-kindled rats treated with PB.

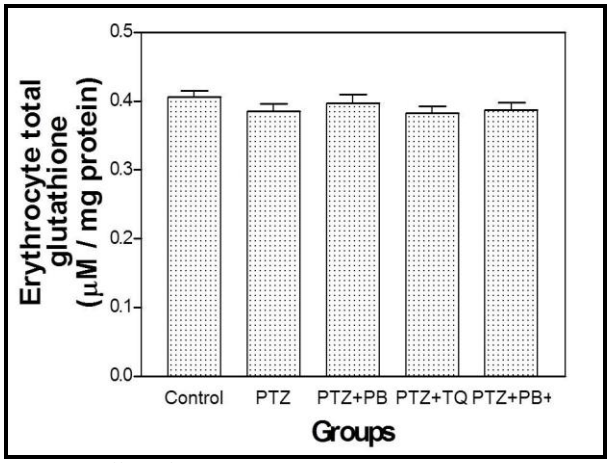

Figure 3. Effect of phenobarbital (PB), thymoquinone (TQ) or their combination on erythrocyte by total glutathione level in PTZ-kindled rats. Results are expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post-hoc test at $P < 0.05$. [a]Significantly different from the control group, [b]significantly different from PTZ-kindled rats, and [c]significantly different from PTZ-kindled rats treated with PB.

significant increase in erythrocyte oxidized glutathione (GSSG) levels in PTZ-kindled rats and PTZ-kindled rats treated PB alone than control rats. Meanwhile, the treatment of the PTZ-kindled rats with PB showed significant decrease in GSSG level than PTZ-kindled group. However, erythrocyte GSSG levels in kindled rats treated with thymoquinone alone or the combination PB and thymoquinone showed significant decrease in GSSG levels than PTZ-kindled and PTZ-kindled treated by PB group.

The activity of erythrocyte GPx has been demonstrated in Fig.5. A significant increase in GPx activity was observed in all PTZ-kindled rats than control group

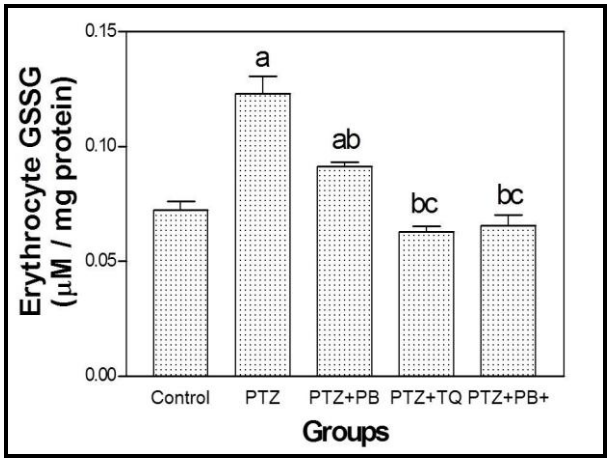

Figure 4. Effect of phenobarbital (PB), thymoquinone (TQ) or their combination on erythrocyte GSSG levels in PTZ-kindled rats. Results are expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post-hoc test at $P < 0.05$. [a]Significantly different from the control group, [b]significantly different from PTZ-kindled rats, and [c]significantly different from PTZ-kindled rats treated with PB.

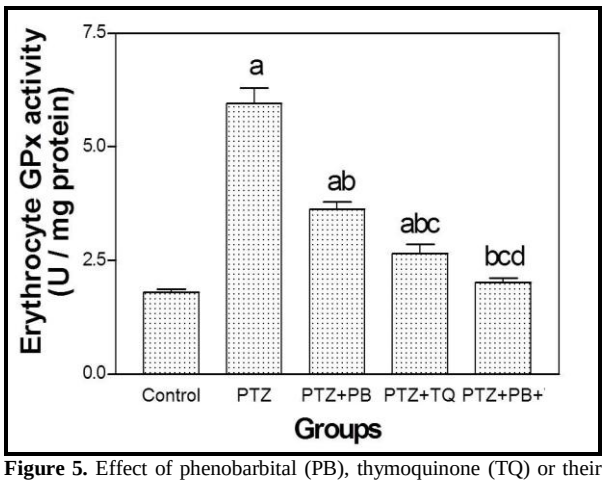

Figure 5. Effect of phenobarbital (PB), thymoquinone (TQ) or their combination on erythrocyte GPx activity in PTZ-kindled rats. Results are expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post-hoc test at $P < 0.05$. [a]Significantly different from the control group, [b]significantly different from PTZ-kindled rats, [c]significantly different from PTZ-kindled rats treated with PB, and [d]significantly different from PTZ-kindled rats treated with TQ.

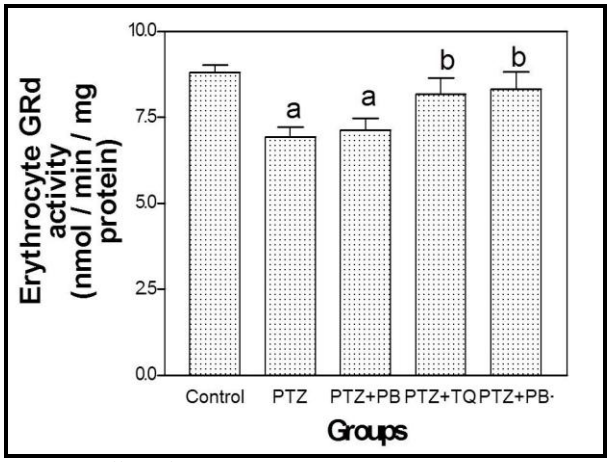

Figure 6. Effect of phenobarbital (PB), thymoquinone (TQ) or their combination on erythrocyte GRd activity in PTZ-kindled rats. Results are expressed as mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's post-hoc test at $P < 0.05$. [a]Significantly different from the control group, and [b]significantly different from PTZ-kindled rats.

except with the combination of PB and thymoquinone, while treatments by PB or thymoquinone significantly decrease erythrocyte GPx activity than PTZ-kindled rats. A further significant decrease in GPx activity was observed in rats treated by thymoquinone, or the combination than the rats treated by PB alone. Also, the combination shows significant decrease in GPx activity than that in the group treated with thymoquinone.

Contrary to the previous results, Fig.6 demonstrated a significant decrease in erythrocyte GRd activity in PTZ-kindled rats and PTZ-kindled rats treated with PB alone if compared to the control group. On the other hand, treatment by thymoquinone or addition of thymoquinone to PB significantly increased erythrocyte GRd activity more in the PTZ-kindled group GRd activity which returned to normal.

DISCUSSION

The present study showed that the combination of phenobarbital and thymoquinone had additive anticonvulsant effect compared to monotherapy with phenobarbital. In addition, although treatment with PB alone showed a significant improvement in plasma MDA level, erythrocyte GSH level, and erythrocyte GPx activity than PTZ-kindled rats, combination of PB with thymoquinone showed more significant improvement in plasma MDA level, erythrocyte GSH level, GPx activity, and erythrocyte GRd activity compared to PTZ-kindled rats or rats received single treatment with PB.

Chronic *in vivo* models of epilepsy provides a suitable strategy for quantifying epileptogenesis, as well as evaluating neuronal plasticity associated with long-term alterations in neural excitability [20]**.** Kindling is a

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well-known model of epilepsy which was first described by Goddard [31], as the repeated application of initially subconvulsive electrical stimulation of different brain structures, results in a progressive development of generalized tonic-clonic seizures**.** Kindling can also be obtained by administration of subconvulsive doses (30-35 mg/kg) of PTZ [32-34].

After triggering these stimuli repeatedly, the response to the stimulus increases. Eventually the stimulus leads to maximal stage of seizures and it will continue to elicit a seizure if triggered subsequently [35]**.** Once such seizures they established, appear to be permanent for the life of the animal [20] and therefore, presumably associated with chronic changes in neuronal excitabilities [35]. This property is used as an experimental animal model of epilepsy and epileptogenesis [36] **.**

The development of PTZ kindling may be related to functional alteration in various neurotransmitter systems, including a gradual reduction in $GABA_A$ receptor function and enhancement of glutamatergic transmission that together result in hyper-excitability and seizure activity [37]**.** Investigations concerning the biochemistry of glutamate, especially modifications in glutamate binding, showed increased glutamate release and increased receptor density in target neuron populations [38] after either electrical [39] or chemical kindling with PTZ in rats [40] or mice [41].

Increase in extracellular glutamate concentration, a process called excitotoxicity, leads to a complex series of biochemical events leading to neuronal injury [42]**.** Various mechanisms for the genesis of seizures have been proposed; however, over-excitation of the excitatory amino acid glutamate, and inhibition of GABAergic system has gained much acceptance [43]. Kindling reflects an altered activity of the excitatory glutamatergic synaptic processes [32, 44]**.** Majority of studies have shown increased glutamate ligand binding in PTZ-kindled animals [32, 40, 41, 45], although possibly accompanied by a down-regulation of glutamate receptor gene expression in several areas of the kindled brain [45]. These results have been interpreted as an enhancement of glutamatergic neurotransmission during the kindling process, which led to decreased seizure threshold [46].

Among different glutamate receptors, N-methyl-d-aspartate (NMDA) receptors were particularly investigated. The effectiveness of different NMDA receptor antagonists as anticonvulsants in several seizure models and in retarding PTZ-kindling proved the role of NMDA-mediated glutamatergic neurotransmission in the increased neuronal excitability during kindling and led to the suggestion of their potential clinical utility in the prophylaxis of epilepsy [33, 44, 47]**.** Unfortunately, NMDA receptor antagonists induce severe neurotoxicity, which limits its clinical use [48]**.** The $GABA_A$ receptor contains a binding site for the endogenous ligand GABA, convulsants (picrotoxin and t-butylbicyclophosphorothionate) binding site, as well as a number of modulatory sites that recognize barbiturates, neurosteroids, ethanol, and benzodiazepines [49].

During kindling, GABAergic receptor function may be decreased, although this may be secondary to the changes in glutamatergic neurotransmission. PTZ interacts competitively with the picrotoxin-binding site of the $GABA_A$ receptors, thereby decreasing chloride flux across the membrane and induces generalized tonic-clonic seizures [36]**.** Previous pharmacological evidence showed that compounds, which modulate GABAergic function via the $GABA_A$ receptors, decrease the susceptibility to PTZ-induced seizures in rats [50, 51] and mice [41]**.** Likewise, the well-known effect of PB in facilitating GABAergic transmission is likely to contribute to its antiepileptogenic effect in the kindling model [20]**.**

In nervous system, the phenomena denominated excitotoxicity have been related to over-production of free radicals by the tissues. The excessive release of excitatory aminoacids, such as glutamate, may kill neurons following excessive activation of their receptors. Moreover, glutamate impairs calcium homeostasis thus, inducing oxidative stress [52]. The activation of glutamate receptors increases the flux of calcium intracellularly. The increase in intracellular calcium, the hallmarks of excitotoxicity, has been documented during epileptiform events in various models of epilepsy, including PTZ-kindling model [10, 52, 53]. Free radicals have been reported to generate due to the glutamate-mediated increase of calcium ions through various mechanisms including mitochondrial dysfunction and activation of nitric oxide synthase [17]. A previous study indicated that there is a linkage between the generation of tonic-clonic seizures and the increased formation of hydroxyl radicals ($^{\bullet}OH^{-}$) in the brain. Shortly after PTZ application, a significant enhancement of $^{\bullet}OH^{-}$ production in PTZ-kindled animals was observed. This observation indicates an important role for $^{\bullet}OH^{-}$ generation as a process mediating neuronal death during kindling. During PTZ-kindling, possible source of $^{\bullet}OH^{-}$ is the peroxynitrite anion ($ONOO^{-}$), which is generated by the spontaneous reaction of the superoxide anion radical ($O_2^{\bullet-}$) and the nitric oxide radical ($NO^{\bullet}$), but rapidly decomposes at physiological pH to yield $^{\bullet}OH^{-}$. Hence, when $NO^{\bullet}$ synthesis is enhanced, *e.g.* during seizures, the formation of $^{\bullet}OH^{-}$ is favored strongly. $^{\bullet}OH^{-}$ initiates processes of LPO, which in turn induces cell damage [35]. This came in line with the increased plasma MDA

levels in the present study. Our data demonstrated that oxidative stress has occurred after PTZ-kindling. The increased MDA levels in the plasma following seizure induction strongly suggest a major role for free radicals in the kindling procedures. Similarly, a previous result indicated that lipid peroxidation occurs in hippocampus after status epilepticus induced by pilocarpine or kainic acid in rats [10]. In addition, a previous report indicated that LPO has been increased during seizures in PTZ-kindled rats [54]. Their data confirm and extend the circumstantial evidences from past studies, which suggested that oxidative stress might occur during seizures and participate in the pathophysiology of epilepsy.

In current model, PTZ-kindled rats showed an elevated erythrocyte GPx activity, which took place as a response to oxidative stress. This data suggested that hydrogen peroxide (H_2O_2) and other peroxides are the major reactive oxygen species produced in the present model. This result agrees with that of Layton and Pazdernik [55]. Therefore, GPx was expressed to overcome these peroxides. In addition, previous electrophysiological studies demonstrated that H_2O_2 promoted hyper-excitability in hippocampal cultured slices by reducing inhibitory neurotransmission and by enhancing of the excitatory neurotransmission [56]. This observation augments our understanding that oxidative stress is coupled with excitotoxicity and consequently, the logic use of antioxidants to control seizures.

Nitric oxide is a singling gaseous molecule formed in the brain as a part of normal intracellular calcium signaling, playing highly diversified roles in cellular pathology [57]**.** NMDA receptor activation generates $NO^{\bullet}$ and it was postulated that an overproduction of this molecule is the link between the actions of excitatory amino acids and the subsequent tissue damage during kindling [58]. The role of $NO^{\bullet}$ in epileptogenesis is still unclear; $NO^{\bullet}$ has been suggested to be either an anticonvulsant or a proconvulsive agent [59]**.** The decrease of erythrocyte GRd activity in kindling rats may be attributed to the increase of $NO^{\bullet}$ that is known to inhibit erythrocyte GRd activity [60]**.** Meanwhile, GRd is a member of GRd-GPx system which is considered as an important protector from oxidative damage. The inhibition of erythrocyte GRd activity and stimulation of erythrocyte GPx activity in PTZ-kindled rats may further illustrate the increase in the concentration of erythrocyte GSH. This result came in harmony with another study [61] that indicated a depletion of erythrocyte reduced form glutathione in PTZ-kindled rats.

In the present study, treatment with phenobarbital during kindling procedures depressed very effectively the kindling development. This observation demonstrates the chronic anticonvulsive effect of phenobarbital, mediated through the inhibition of GABAergic transmission. The results presented here substantiates a previous finding which indicated similar influences of phenobarbital on kindling development [20]**.** The antiepileptic activity of phenobarbital may illustrate the significant decrease in plasma MDA levels and erythrocyte GSH concentrations. The results cope with the decrease in erythrocyte GPx activity but not with erythrocyte GRd activity. The previous data further illustrate the significant increase in erythrocyte GSH level in PTZ-kindled rats treated with phenobarbital than that in the control group.

The ability of antioxidant treatment to significantly enhance the effect of phenobarbital in the present model of epilepsy is an agreement with our hypothesis and cope with a recent study, which demonstrated the effect of vitamin E and clobazam on MDA levels in rat brain, and the pattern of electroshock is assessed [61]**.** This may illustrate the significant decrease in plasma MDA levels and erythrocyte GPx activity in kindled rats treated with phenobarbital and thymoquinone than kindled rats treated with phenobarbital alone. Moreover, erythrocyte GSSG concentrations and erythrocyte GRd activity showed no changes in kindled-rats treated with phenobarbital and thymoquinone comparing with control rats.

Finally, our results indicated that phenobarbital alone or in combination with thymoquinone could suppress both seizures and PTZ-induced lethality in kindled rats. This conclusion is supported by the earlier results of Ullah *et al* [62] which reported that diazepam and valproic acid could suppress tonic seizures and lethality induced by PTZ in kindled mice. On the other hand, thymoquinone in combination with phenobarbital seem to have significant antioxidant activities and may have a neuroprotective property in this experimental model. This conclusion is supported by a recent study which indicated that thymoquinone has a neuroprotective effect [63]**.** On the other hand, this combination has no significant anticonvulsant properties than phenobarbital alone.

In conclusion, the data represented in this study suggested that thymoquinone may have a significant anticonvulsant and antioxidant effect in the current model of chronic epilepsy when combined with phenobarbital. Further studies will be needed.

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