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Ameliorative Potential of Resveratrol on Experimentally-Induced Seizures

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Abstract The aim of this study was to investigate the potential enhancement effects of resveratrol on the anticonvulsant effect of diazepam in pilocarpine induced seizures in rats. This study was carried out in 50 male albino rats subdivided into five equal groups; group1 received saline 0.9 % intraperitoneally and served as the control group; group 2 received pilocarpine; group 3 received resveratrol 30 min prior to pilocarpine; group 4 received diazepam 30 min prior to pilocarpine; group 5 received resveratrol/ diazepam combination 30 min prior to pilocarpine. Then, animals were decapitated and the hippocampus was collected and homogenized for determination of tissue interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), nitric oxide (NO), malondialdehyde level (MDA), superoxide dismutase (SOD) and catalase (CAT) activity. Resveratrol/diazepam combination produced significant decrease in tissue cytokines, NO and MDA while produced significant increase in tissue SOD and CAT activity compared to the use of diazepam alone. Also, this combination produced significant delay in the onset of convulsions and significant decrease in the mortality rates compared to the use of diazepam alone. In conclusion, resveratrol potentiates the anticonvulsant effect of diazepam and this may be beneficial in lowering the incidence of resistance to diazepam and also may allow reduction of its dose and side effects.

Keywords: *resveratrol, diazepam, pilocarpine, seizures*

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1. Introduction

Epilepsy is the most frequent neurodegenerative disease after stroke. It affects more 50 million people worldwide [1]. More than 30% of patients with epilepsy continue to have inadequate control of their seizures despite medical treatment [2]. Currently, more than 20 antiepileptic drugs (AEDs) approved by the U.S. Food and Drug Administration for the treatment of epilepsy. However, all of these medications are associated with side effects including gastrointestinal disturbances, central nervous system symptoms such as somnolence and behavioral disturbances, bone marrow suppression, Stevens-Johnson syndrome and teratogenicity [3]. Though several AED combinations are useful for patients with refractory epilepsy, many epileptic patients remain refractory to AED combinations. Therefore, a need was felt for some novel combinations of AEDs or a combination of AEDs with natural products possessing anti-seizure activity for suppressing of epileptic attacks [4].

Experimental models of seizures and epilepsy have been valuable for understanding of the basic mechanisms underlying pathogenesis of epilepsy as well as for developing new therapeutic options [5]. One of the earliest and most frequently used models for evoking experimental status epilepticus and subsequent epilepsy is the systemic injection of pilocarpine in rodents. Pilocarpine is a cholinergic agonist used for developing experimental models of intractable epilepsy with seizures resembling status epilepticus or complex partial seizure in humans [6].

Many evidences support the hypothesis that experimental epilepsy is mediated by oxidative stress. Also, it is known that oxidative stress occurs as a result of seizures [7]. The high rate of oxidative metabolism, coupled with the low antioxidant defenses and the richness in polyunsaturated fatty acids, makes the brain highly vulnerable to damage induced by free radicals [8].

Clinical and experimental studies have provided proof-of-concept evidence that brain inflammation is an important factor in epilepsy. In particular, high levels of proinflammatory cytokines such as interleukin (IL)-1 beta and tumor necrosis factor alpha (TNF-α) [9]. The involvement of cytokines in the pathogenesis of epilepsy has been suggested by the evidence that limbic seizures increase messenger RNA (mRNA) of the inflammatory cytokines in rodent forebrain [10]. In addition, the release of TNF-α and IL-6 from rat hippocampal slices is enhanced by seizures. Moreover, cytokine expression in immature brain is associated specifically with cell injury rather than with seizures, suggesting that proinflammatory

cytokines may contribute to the occurrence of status epilepticus induced hippocampeal damage [11].

Resveratrol is a non-flavonoid polyphenolic compound found in grapes and the red wine prepared from them. Resveratrol shows pleiotropic health beneficial effects, including anti-oxidant, anti-inflammatory, anti-aging, cardioprotective and neuroprotective activities [12]. Resveratrol has been shown to modulate lipoprotein metabolism, eicosanoid synthesis, lipid peroxidation, and platelet aggregation [13]. Resveratrol also suppresses the induction of nitric oxide synthase and disrupts arachidonic acid metabolism by inhibiting cyclooxygenase-2 [14]. The ability to enter the brain after a peripheral administration and no adverse effects on the brain or body are other features that are appealing for using this compound as a therapy for brain injury or neurodegenerative diseases [15].

Based on the above considerations, the aim of this study was to evaluate the potential enhancement effects of the natural antioxidant anti-inflammatory drug (resveratrol) on the anticonvulsant effect of one of established commonly used drugs (diazepam) on pilocarpine induced seizure in rats in an attempt to increase the efficiency and decrease the dose and side effects of diazepam.

2. Materials and Methods

2.1. Chemicals

All chemicals and kits used in this study were purchased from Sigma chemical CO., USA.

2.2. Drugs

- Pilocarpine hydrochloride was purchased from Sigma chemical CO., USA.

- Resveratrol was purchased from the Sigma Chemical Co. and was dissolved in normal saline and given in a dose of 40 mg/kg intraperitoneal [16].

- Diazepam was purchased from Roche/Egy Co. and given in a dose of 10 mg/kg intraperitoneal [17].

2.3. Experimental Design

Fifty albino rats weighing 150 – 200 grams were used throughout this study. Animals were housed in standard cages under a12-hour light/12-hour dark cycle and had free access to food and water. All the experiments were conducted according to the National Research Council's guidelines. Animal handling was followed according to Helsinki declaration of animal ethics. Animals were subdivided into five equal groups as follows:

Group 1: Rats were given saline 0.9 % intraperitoneally and served as the control group.

Group 2: Rats were given pilocarpine dissolved in normal saline and the pH adjusted to 7.4 and was given in a single dose of 400 mg intraperitoneal [18].

Group 3: Received resveratrol single dose of 40 mg/kg intraperitoneal administered 30 min prior to pilocarpine [16].

Group 4: Received diazepam single dose of 10 mg/kg intraperitoneal administered 30 min prior to pilocarpine [17].

Group 5: Received diazepam and resveratrol administered 30 min prior to pilocarpine in doses as given above.

Rats were put in individual cages and observed for 24 hours following pilocarpine injection for behavioral changes. The behavioral changes observed were similar to those recorded by Turski et al. [19] where animals were motionless for 5–10 minutes after pilocarpine administration and subsequently displayed oro-facial movements, salivation, eye-blinking, twitching of vibrissae and yawning. This activity persisted up to 45 minutes. Then, discontinuous seizures were observed 30 minutes after injection and lasted up to 90–150 minutes, before giving way to limbic motor seizures with intense salivation, rearing, upper extremity clonus and falling. Such seizures were observed every 5–15 minutes, presenting maximal frequency after 1–2 hours. Status epilepticus spontaneously remitted 5–6 hours after pilocarpine administration and the animals entered post-ictal coma, lasting for 1 day. The time from injection of each drug to the first appearance of seizure activity was measured for each animal and is referred to as the seizure latency. Only animals with seizure activity were considered to calculate the latency to the onset of seizures. The latency to death was observed in the cut-off 24 hours. Then, animals were decapitated under ether anesthesia and the hippocampus was collected and homogenized in 2 volumes of chilled 50 Mm potassium phosphate buffer PH 7.0 for further assay. Homogenates were centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatant was transformed to chilled Eppendorf tubes and used for determination of the following parameters:

- Interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) using ELISA kits purchased from Sigma chemical Co.
- Tumor necrosis factor alpha (TNF-α) using ELISA kits supplied by RayBiotech, Inc. according to Kabel et al. [20].
- Nitric oxide measurement was done by ELISA using total NO/Nitrite/Nitrate assay [21].
- Malondialdehyde level was measured based on method of Draper and Hadley [22].
- Superoxide dismutase activity assay was analyzed using the method of Tsai et al. [23].
- Catalase activity assay was analyzed using the methods of Aebi [24].

2.4. Statistical Analysis

Results were expressed as the mean ± SEM. Comparison between different groups was carried out by one-way analysis of variance test (ANOVA) followed by LSD test. The statistical significance was accepted at a level of p-value less than 0.05.

3. Results

3.1. Effect of the Different Treatments on the Incidence and Onset of Convulsions

Administration of pilocarpine produced significant increase in the number of animals having tonic/clonic convulsions and significant increase in the onset of convulsions compared to the control group. Administration of resveratrol or diazepam prior to pilocarpine produced significant decrease in the number of

animals having tonic/clonic convulsions and significant delay in the onset of convulsions compared to the pilocarpine group. Administration of resveratrol/diazepam combination produced significant decrease in the number of animals having tonic/clonic convulsions with significant delay in the onset of convulsions compared to the pilocarpine group.

3.2. Effect of the Different Treatments on the Interval until Death

Administration of pilocarpine produced significant decrease in the interval until death compared to the control group. Administration of resveratrol or diazepam prior to pilocarpine produced significant increase in the interval until death compared to the pilocarpine group. Administration of resveratrol/diazepam combination produced significant increase in the interval until death compared to the pilocarpine group.

3.3. Effect of the Different Treatments on Tissue Cytokines

Administration of pilocarpine produced significant increase in tissue IL-1β, IL-6 and TNF-α compared to the control group. Administration of resveratrol produced significant decrease in tissue IL-1β, IL-6 and TNF-α compared to the pilocarpine group. Administration of diazepam produced non-significant effect on tissue IL-1β, IL-6 and TNF-α compared to the pilocarpine group. Administration of resveratrol/diazepam combination produced significant decrease in tissue IL-1β, IL-6 and TNF-α compared to the pilocarpine group.

3.4. Effect of the Different Treatments on Tissue NO and MDA

Administration of pilocarpine produced significant increase in tissue NO and MDA compared to the control group. Administration of resveratrol produced significant decrease in tissue NO and MDA compared to the pilocarpine group. Administration of diazepam produced non-significant effect on tissue NO and MDA compared to the pilocarpine group. Administration of resveratrol/ diazepam combination produced significant decrease in tissue NO and MDA compared to the pilocarpine group.

3.5. Effect of the Different Treatments on Tissue Antioxidant Enzymes

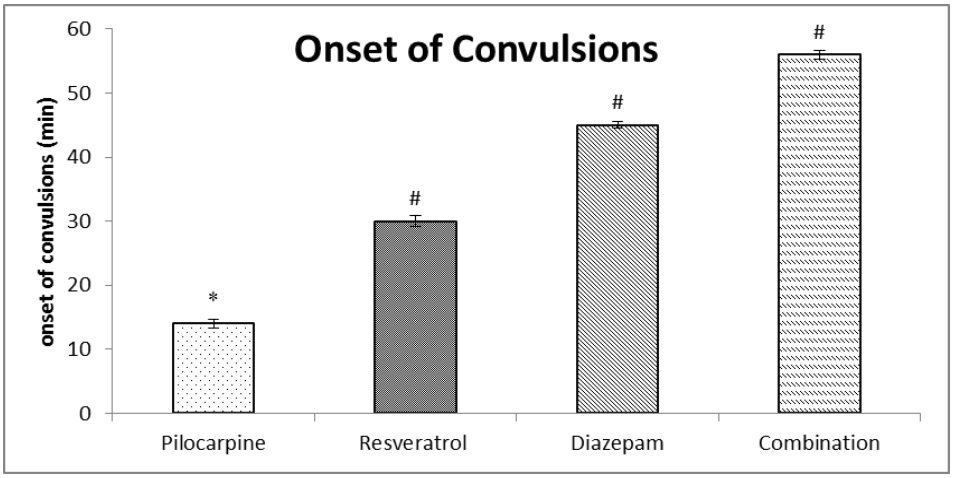

Figure 1. Effect of different treatments on the onset of convulsions

* Significant when compared to control group (P<0.05).
# Significant when compared to pilocarpine group (P<0.05).

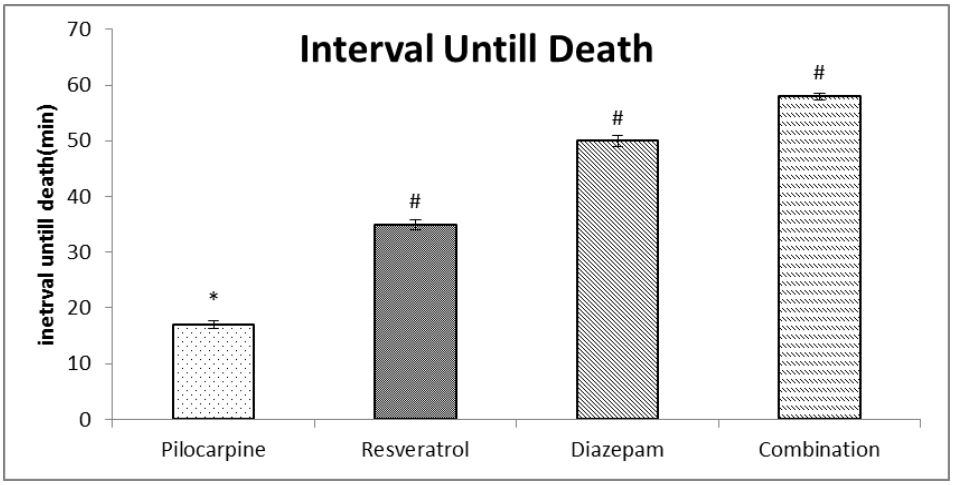

Figure 2. Effect of different treatments on the interval until death

*Significant when compared to control group (P<0.05).
Significant when compared to pilocarpine group (P<0.05).

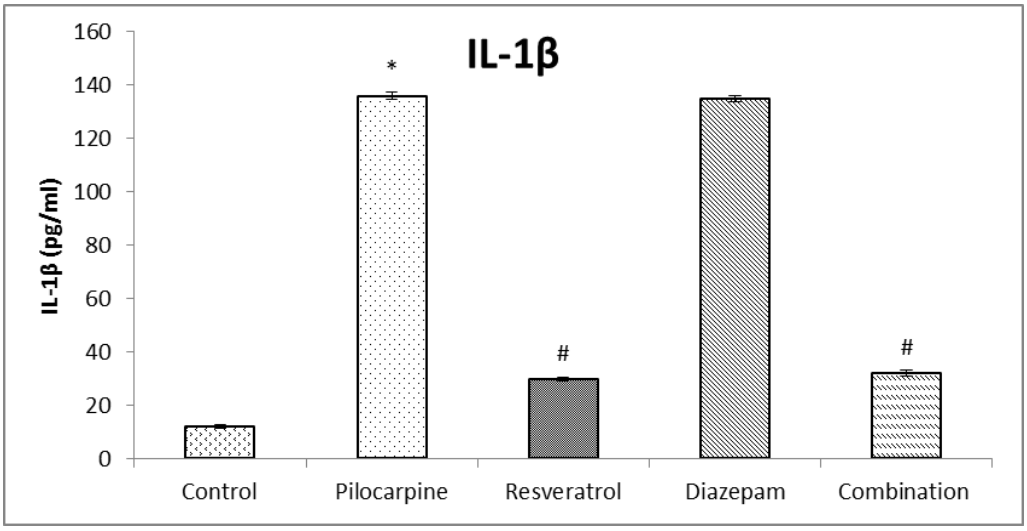

Figure 3. Effect of different treatments on tissue IL-1β

* Significant when compared to control group (P<0.05).
# Significant when compared to pilocarpine group (P<0.05).

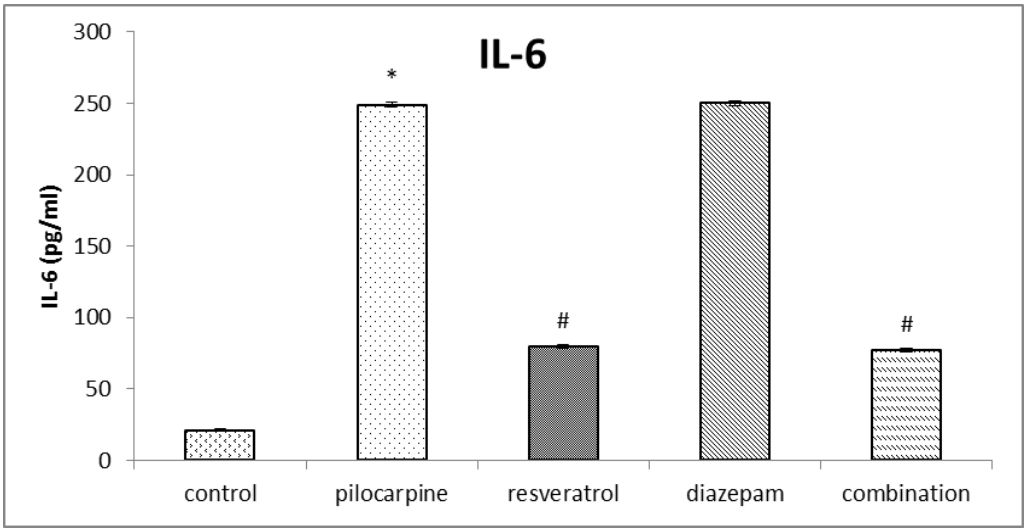

Figure 4. Effect of different treatments on tissue IL-6

*Significant when compared to control group (P<0.05).
Significant when compared to pilocarpine group (P<0.05).

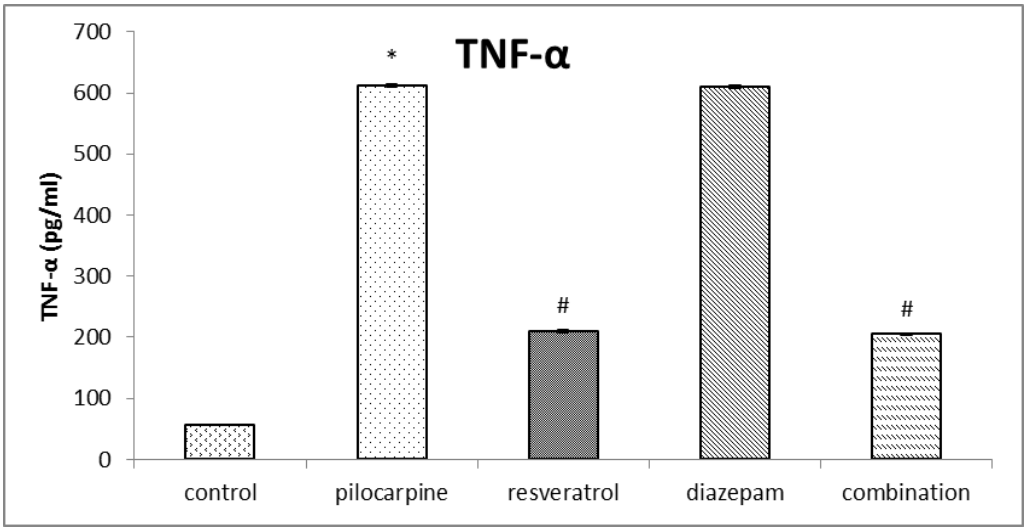

Figure 5. Effect of different treatments on tissue TNF-α

*Significant when compared to control group (P<0.05).
Significant when compared to pilocarpine group (P<0.05).

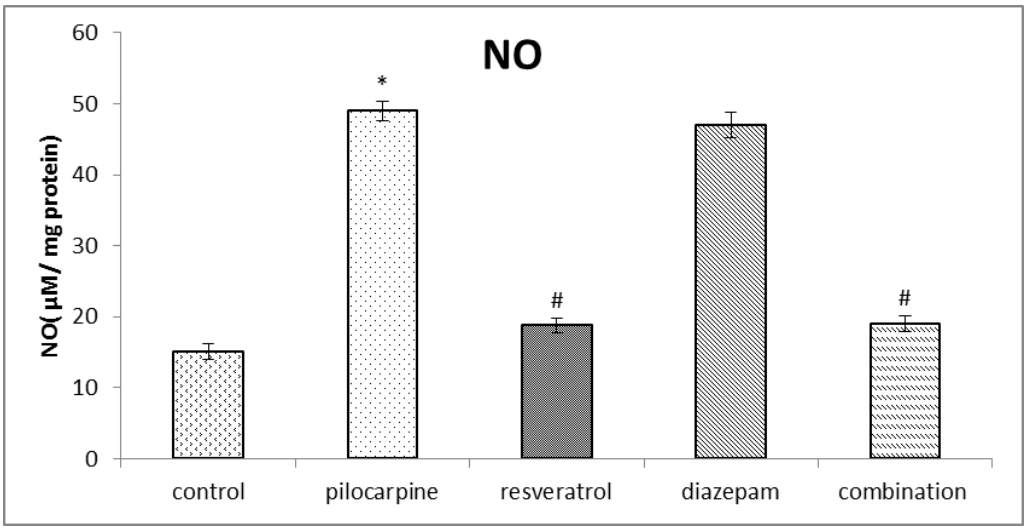

Figure 6. Effect of different treatments on tissue NO

*Significant when compared to control group (P<0.05).
Significant when compared to pilocarpine group (P<0.05).

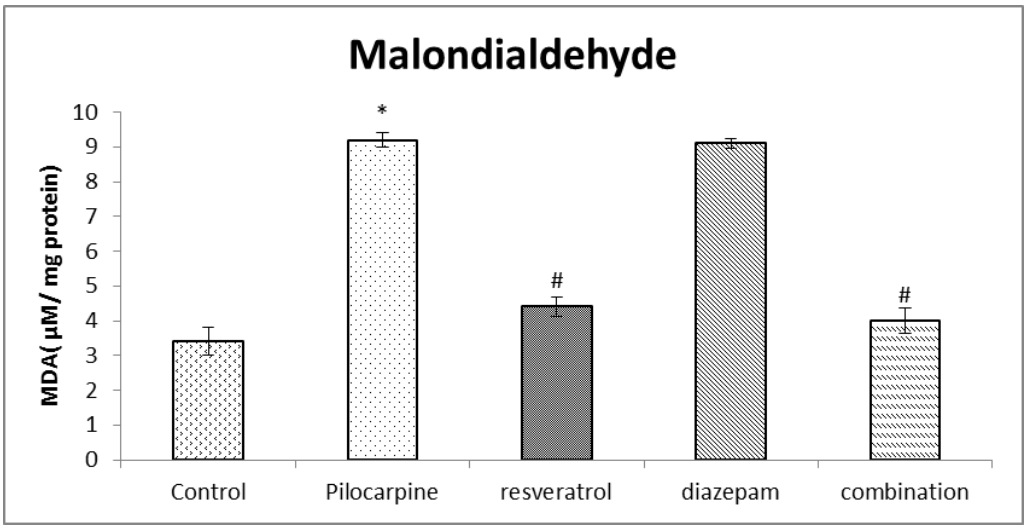

Figure7. Effect of different treatments on tissue MDA

*Significant when compared to control group (P<0.05).
Significant when compared to pilocarpine group (P<0.05).

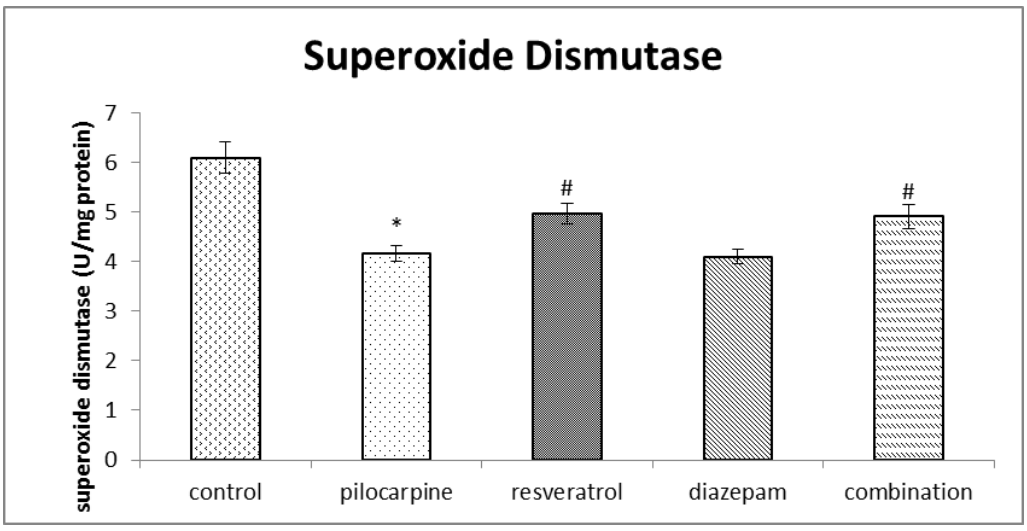

Figure 8. Effect of different treatments on tissue superoxide dismutase

* Significant when compared to control group (P<0.05).
# Significant when compared to pilocarpine group (P<0.05).

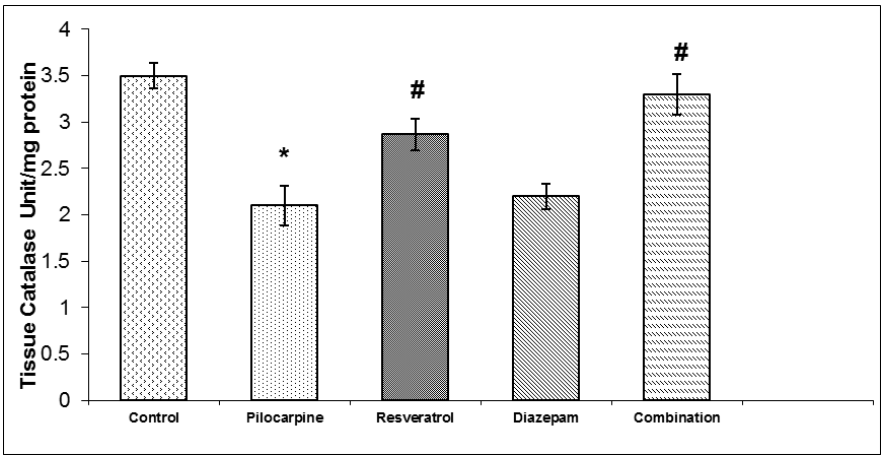

Figure 9. Effect of different treatments on tissue catalase

* Significant when compared to control group (P<0.05).
# Significant when compared to pilocarpine group (P<0.05).

Administration of pilocarpine produced significant decrease in tissue superoxide dismutase and catalase compared to the control group. Administration of resveratrol produced significant increase in tissue superoxide dismutase and catalase compared to the pilocarpine group. Administration of diazepam produced non-significant effect on tissue superoxide dismutase and catalase compared to the pilocarpine group. Administration of resveratrol/diazepam combination produced significant increase in tissue superoxide dismutase and catalase compared to the pilocarpine group.

4. Discussion

The anticonvulsive mechanisms of the conventional and the newly introduced drugs vary considerably. The most common actions were shown on ion channels, GABA and glutamate metabolism, receptors or secondary messengers [25]. Extensive efforts have been made to achieve neuroprotection through effective seizure suppression with anticonvulsants and new compounds that may be neuroprotective through mechanisms other than anticonvulsant actions. Neuronal hyperexcitability and excessive production of free radicals have been implicated in the pathogenesis of a considerable range of neurological disorders, including epilepsy. The high rate of oxidative metabolism, coupled with the low antioxidant defenses and the richness in polyunsaturated fatty acids, makes the brain highly vulnerable to free radical damage [8].

In the present study, intraperitoneal administration of pilocarpine produced significant increase in number of animals having tonic/clonic convulsions together with significant increase in mortality rate. This is in accordance with Jope et al. [26] who reported that pilocarpine single dose of 400 mg intraperitoneally induce status epilepticus (SE) in 83% of animals and mortality was 100%. Moreover, Curia et al. [27] stated that mortality increased to 50% and 100% with 350 and 400 mg/kg of pilocarpine, respectively with occurrence of motor limbic seizures more rapidly in the animals treated with the highest pilocarpine dose.

The ability of pilocarpine to induce SE is likely to depend on activation of the M1 muscarinic receptor subtype, since M1 receptor knockout mice do not develop seizures in response to pilocarpine [27]. In addition, pilocarpine-induced SE can be blocked by systemic administration of the muscarinic antagonist atropine [28]. Once seizures are initiated, however, their maintenance depends on other mechanisms since atropine becomes ineffective [27]. Some findings raise the possibility that pilocarpine-induced seizures are not only the result of direct cholinergic system activation in the brain, but also are derived from primary proinflammatory actions of pilocarpine involving the periphery where pilocarpine causes acute peripheral proinflammatory changes manifested by elevated serum levels of IL-1β together with reduction in the number of CD4-expressing cells leading to blood–brain barrier (BBB) leakage, shortly after pilocarpine injection and prior to the onset of status epilepticus [29].

The concept that peripheral inflammatory reactions contribute to lower seizure threshold is in agreement with Harvey and Boksa [30] who stated that systemic lipopolysaccharides (A component of the bacterial wall of Gram-negative bacteria mimicking infection) reduces the threshold to seizures in adult rodents. Further studies demonstrated that lipopolysaccharides or intracolonic administration of 2, 4, 6-trinitrobenzene sulfonic acid (in a model of bowel disease), delivered to immature rats (postnatal day 7 or 14), was reported to induce long-lasting increases in seizure susceptibility and seizure-associated brain damage [31,32].

Regarding to the effect of pilocarpine on inflammatory markers in our present work, it was found that pilocarpine significantly increase tissue levels of IL-1β, IL-6 and TNF-α. This in accordance with results recorded by Vezzani [33] who postulated that IL-1β is elevated in serum early after pilocarpine or lithium chloride administration and considered to be an etiological factor in BBB breakdown. Moreover, IL-1β possesses proconvulsant activity when applied to the brain [34]. This was supported by Marchi et al. [5] who postulated that antagonism of peripheral inflammation reduces the severity of status epilepticus. Several studies reported increased expression of mRNA for IL-1β, IL-6, iNOS and TNF-α after seizures. Ravizza el al. [35] showed that specific inflammatory pathways are chronically activated during epileptogenesis and they persist in chronic epileptic tissue, suggesting that they may contribute to the pathogenesis of TLE. Increased level of NO after pilocarpine injection in our work was in the same line with Hrncic et al. [36] that emphasized that

administration of 7-Nitroindazole, an inhibitor of neuronal nitric oxide synthase, attenuates pilocarpine-induced seizures.

In the present work, administration of pilocarpine induced significant decrease in antioxidant parameters represented by decrease in tissue catalase and superoxide dismutase and this was accompanied by significant elevation in MDA and NO levels. This is in agreement with Tsai et al. [22] who recorded that rats received pilocarpine show significant increase in MDA level together with significant decrease in catalase, superoxide dismutase levels. On the other hand, Dos Santos et al. [37] stated that rats injected with pilocarpine showed a significant increase in lipid peroxidation and nitrite levels together with increase the catalase activity with insignificant changes on superoxide dismutase tissue level. Increased inflammatory markers have been detected in serum, CSF, and brain of people with epilepsy. There are relevant findings of increased IL-6 following recent tonic-clonic seizures [38,39].

In the present work, administration of resveratrol induced significant decrease in number of animals having tonic/clonic convulsion with significant decrease in mortality rate and increase in latency to convulsion. These results were accompanied by significant reduction in inflammatory markers IL-1β, IL-6 and TNF-α. This is in accordance with Wu et al. [40] who stated that resveratrol decreased the frequency of spontaneous seizures and inhibited the epileptiform discharges when applied to kainate-induced temporal lobe epilepsy (TLE) in rat.

Inhibition of the lipid peroxidation by resveratrol has been demonstrated in several studies [40,41] who demonstrated that intraperitoneal administration of resveratrol in a healthy normal rat decreased brain malondialdehyde (MDA) levels and increased brain superoxide dismutase and catalase activities. Moreover**,** Grissa et al. [42] showed that resveratrol reduced lipid peroxidation as indicated by decrease in MDA levels to nearly those measured in control rats when compared with rats injected with ethanol alone intraperitoneally.

Another study using neuronal cell cultures demonstrated that resveratrol treatment induces heme oxygenase 1 activity with no detectable toxic effects [43]. Because heme levels increase inside cells after stroke and heme (iron-protoporphyrin IX) is considered a pro-oxidant, its rapid degradation by heme oxygenase is believed to be neuroprotective. From this perspective, increased heme oxygenase activity is likely one of the mechanisms by which resveratrol functions as a neuroprotective compound. Thus, resveratrol exerts neuroprotective properties by regulating several detoxifying enzymes. A study examined the effects of resveratrol administration on nitric oxide and tumor necrosis factor-alpha production in cultured microglia that are activated through lipopolysaccharide (LPS) treatment [44]. While the microglial cultures exposed to LPS alone exhibited increased levels of TNF-alpha and NO, microglial cultures exposed to LPS and RESV displayed no significant increases in TNF-alpha and NO. Similar results were recorded by Lu et al. [45] who found that LPS stimulated the expression of TNF-alpha, IL-1beta, IL-6,and iNOS in murine microglia and astrocytes and the use of resveratrol was associated with inhibition of LPS-induced expression and release of TNF-alpha, IL-6 and iNOS/NO in both cell types with more potency in microglia, and inhibited LPS-induced expression of IL-1beta in microglia but not astrocytes.

In the present study, administration of diazepam produced significant decrease in number of animals having tonic/clonic convulsion with significant decrease in mortality rate and increase in latency to convulsion but showed insignificant changes in tissue levels of inflammatory markers (IL-1B, IL-6 and TNF-A., MDA, NO) and the antioxidant parameters (Catalase and superoxide dismutase). This is in accordance with Pitkanen et al. [46] who reported that administration of diazepam reduced the percentage of epileptic animals. Bianchi [47] explained the mode of action of diazepam by modulating the GABA-A receptor**.** First, they increase the frequency of opening of the associated chloride ion channels and hyperpolarize the membrane. These changes facilitate the inhibitory effects of the available GABA and lead to sedation and anxiolytic effects. Second, different benzodiazepines can have different affinities for GABA-A receptors [48]. On the contrary, Paul et al. [49] reported an increase in activity of nitric oxide synthase (NOS) and the concentration of NO in rat brain 15 min after administration of anticonvulsant doses of diazepam (which is known to activate GABA-A receptor), diazepam enhanced both NOS activity and the concentration of NO in a dose-dependent manner. Another study was reported by Seçkin et al. [50] who stated that chronic administration of diazepam caused an increase in malondialdehyde (MDA) levels and a decrease in glutathione (GSH) content in rat liver.

5. Conclusion

The ability of the antioxidants to reduce the seizure manifestations and the accompanying biochemical changes further supports the role of free radicals in seizures and highlights a possible role of antioxidants as adjuncts to antiepileptic drugs for better seizure control. Our findings strongly support the hypothesis that oxidative stress occurs during pilocarpine-induced seizures, indicating that brain damage induced by the oxidative stress plays a crucial role in seizures pathogenic consequences, which implies that the protective effect could be achieved using antioxidants like resveratrol. Hence, resveratrol may have ameliorative effect against prolonged-seizure-induced neuronal death. Resveratrol potentiated the anticonvulsant effect of diazepam and this may be of benefit in lowering the incidence of resistance to diazepam and may allow decrease its dose and side effects.

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