



# Stress as a Risk Factor in Induction and Progression of Alzheimer's Disease: Impact on the Possible Protection Using Epigallocatechin-3-Gallate and/or Diazepam

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## Abstract

**Background/Objective:** Alzheimer's disease (AD) is a progressive neurodegenerative disease. Stress is implicated in the development of AD since oxidative stress has been linked to cognitive impairment. Epigallocatechin-3-Gallate (EGCG) is the most abundant catechin in green tea and has antioxidant, anti-inflammatory and anti-atherogenic effects, while Diazepam is an anxiolytic with promising neuroprotective properties. The study aimed to evaluate the impact of stress on behavioral, biochemical and histopathological changes accompanied induction and progression of AD as well as the possible protection using EGCG and/or Diazepam.

**Methods:** Seven groups (8 rats/group) were daily IP injected for six week either with saline for control (2 groups) or with 70 mg/kg AlCl<sub>3</sub> for AD-induced model (5 groups). Stress was induced for all groups except one control and one AlCl<sub>3</sub> group by exposing rats 6 times during six weeks to Stress-induced box paradigm (one time/week for 30 minute). Three groups of AD-induced model were also daily received either EGCG (10 mg/kg, IP), Diazepam (0.1 mg/kg, IP) or their combination. All rats were examined in two behavioral experiments; Morris water maze task and Conditioned-avoidance test. Histological examination was achieved in different brain regions and biochemical measurements as brain cholinergic markers (AChE); oxidative stress markers (SOD, GPx, MDA, TAC) and inflammatory mediators (TNF- $\alpha$ , IL-1 $\beta$ ) were also assayed for all groups.

**Results:** Rats exposed to AlCl<sub>3</sub> together with stress showed marked decline in learning and memory abilities. Stress also induced significant elevation in hippocampus TNF- $\alpha$ , IL-1 $\beta$  and MDA level as well as in AChE activity accompanied by reduction in GPx, TAC and SOD activities. Marked histopathological brain degenerations were also shown in AD-model group exposed to stress. EGCG showed more marked protective effect than Diazepam from stress-potentiated the deleterious effect of AlCl<sub>3</sub> on the brain, however Co-administration of both resulted in more pronounced protection as regarding all measured parameters.

**Conclusions:** Exposure to stress represents a risk factor in induction and progression of AD. The deleterious effect of stress on the brain and hippocampus can be counteracted by Co-administration of both EGCG and Diazepam.

**Keywords:** Alzheimer's disease; Stress; EGCG; Diazepam; Rats

## Introduction

Alzheimer's disease (AD) is a neurodegenerative condition responsible for the cognitive deterioration of millions of people in the world [1]. AD causes are not fully known and clinical drug trials have a greater than 90% failure rate. There is an urgent need to find accurate methods of early detection as well as effective therapies before patients with AD develop significant brain damage. Clinical diagnosis of probable AD is currently made by excluding other causes using history, exam and labs, structural imaging, and cognitive testing [2].

Stress is believed to contribute to the variability of the ageing process and to the development of age-related neuro- and psychopathologies [3-5]. Clinical data suggest that a stressful lifestyle can be considered a risk factor for AD [6] and stress-related psychiatric disorders as major depression have been identified as a risk for developing AD [7]. There is much interest, therefore, in understanding the mechanisms responsible for interactions among stress, aging and memory. A prim sign of aging is oxidative stress. It has been speculated that the free radicals produced during oxidative stress are

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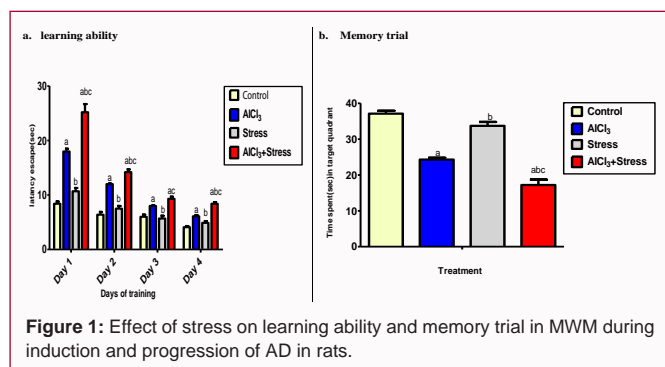
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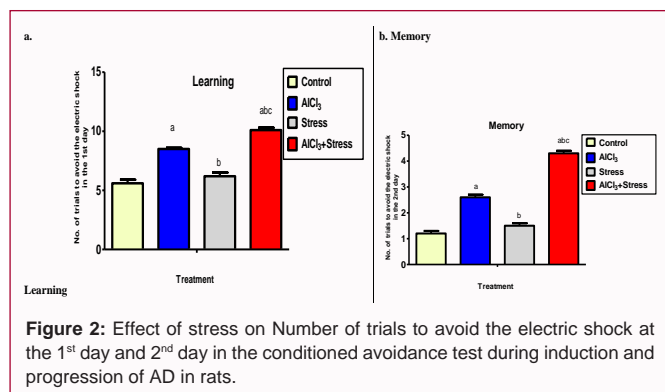
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**Figure 1:** Effect of stress on learning ability and memory trial in MWM during induction and progression of AD in rats.



**Figure 2:** Effect of stress on Number of trials to avoid the electric shock at the 1<sup>st</sup> day and 2<sup>nd</sup> day in the conditioned avoidance test during induction and progression of AD in rats.

pathologically important in AD and other neurodegenerative diseases. Additionally, it has been proposed that not only oxidative stress is a significant early event in the development of the disease, but also it plays an important role in modulating signaling pathways leading to cell death. A study of brains in different disease stages showed increased levels of 4-hydroxyhexenal, a marker of lipid peroxidation, already in the early stages of the disease [8]. Peroxidation of lipids can create electrophilic aldehydes that are highly reactive and easily modify proteins. Acrolein is an example of such an aldehydic lipid peroxidation product that is increased in AD brains [9] and can create damage to mitochondria by inhibiting respiration and modulating tau phosphorylation [10]. Similar observations were made for oxidations of DNA and RNA when measuring levels of 8-hydroxyguanine in hippocampus [11].

Not only are several markers of oxidative stress increased in AD but also there is also evidence for lower antioxidant power in the brain, CSF, and blood. The most prevalent antioxidant in most brain cells is reduced glutathione (GSH). It can react with ROS oxidized products forming glutathione disulphide (GSSG), either catalysed by Glutathione Peroxidase (GPx) or independently. The GSSG can then be converted back to reduced GSH by Glutathione Reductase (GR). Studies of human brains have indicated that the ratio between reduced and oxidized glutathione (GSH/GSSG) is decreased in affected brain regions of AD patients compared to controls [12].

Metal dyshomeostasis has been implicated in many neurodegenerative disorders. Metals play a major catalytic role in the production of free radicals, and attention has centered on the role of many metals in AD, including iron, aluminum, mercury, copper, and zinc [13-15]. Since last three decades, the association between aluminum and AD had gained much interest and it has been shown that aluminum accumulates in all the regions of the brain maximum being in hippocampus, which is the key site of memory and learning. Aluminum has been suggested as a causal factor in

AD, in part because of reports showing the toxicity of aluminum, the elevation of aluminum concentrations in the brains of patients with AD, and an association between aluminum concentrations in water and the prevalence of AD [16-17]. In addition, aluminum is a well-known neurotoxicant reported to accelerate oxidative damage to biomolecules. Furthermore, aluminum salts have been reported to cause cell depletion in the hippocampus [18] and degeneration of cholinergic terminals in the cortical areas. It accumulates in the cingulate bundle and thereby induces learning deficits [19]. Aluminum oxidative stress and impaired cholinergic function have been all related to cognitive dysfunction. Aluminum crosses the blood brain barrier *via* the specific high affinity receptors for transferring [20]. Upon entering the brain it affects the slow and fast axonal transports, induces inflammatory responses [21], inhibits long-term potentiation, and causes synaptic structural abnormalities, thereby resulting in profound memory loss.

Tea is the most consumed drink in the world after water. The majority of tea beverage is prepared from three types of manufactured tea: green tea, oolong tea, and black tea all come from the leaves of the *Camellia sinensis* plant [22]. The secret of green tea lies in the fact it contains high quantities of pharmacologically active compounds. The active constituents are polyphenols, contributing to approximately 30% of green tea Epicatechin (EC), EC Gallate (ECG), Epigallocatechin (EGC), and EGC Gallate (EGCG) [22]. EGCG is the most abundant and active compound responsible for most of green tea's role in promoting good health by acting through different pathways, as antioxidant, anti-inflammatory and anti-atherogenic agent, showing gene expression activity, functioning through growth factor-mediated pathways [22]. As AD is a disease of aging which is directly linked to repeated oxidative stress and chronic inflammation [23], therapies that diminish such effects have become an important tool in seeking more effective treatments for AD [24]. New findings also showed that polyphenol as EGCG has function as neuroprotective agent at multiple levels [25]. In general, EGCG has capacity to chelate metal ions and to directly quench free radical species that contribute to oxidative damage [26]. Numerous epidemiological analyses have established a clear relationship between the neuroprotective effects of EGCG and the decreased risk of age-associated neurological dysfunction such as that found in AD [27].

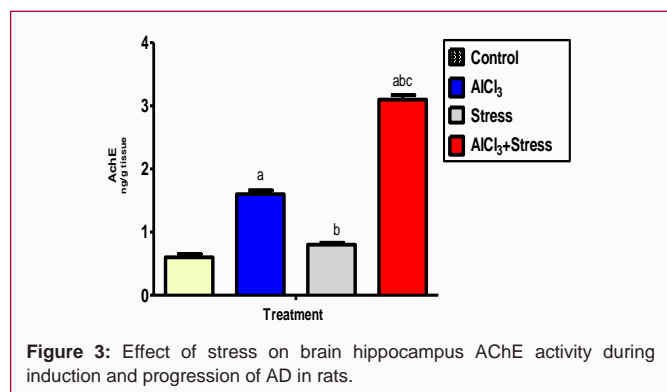
Diazepam is a benzodiazepine with anticonvulsant, anxiolytic, sedative, muscle relaxant, and amnesic properties with a long duration of action. It is the best known benzodiazepine, was developed in the late 1950s and has been marketed as Valium since 1963. It was approved by the FDA in the United States prior to 1982. Although originally intended as an anxiolytic, it now has many other indications. Intravenous diazepam has been used since the 1960s for controlling status epilepticus [28].

Therefore, the aim of this study was to evaluate the potential role of stress on behavioral, biochemical and histopathological changes which accompanied the induction and the progression of AD. It also aimed to study the possible protective effect of EGCG and /or Diazepam against development of AD in stressful condition.

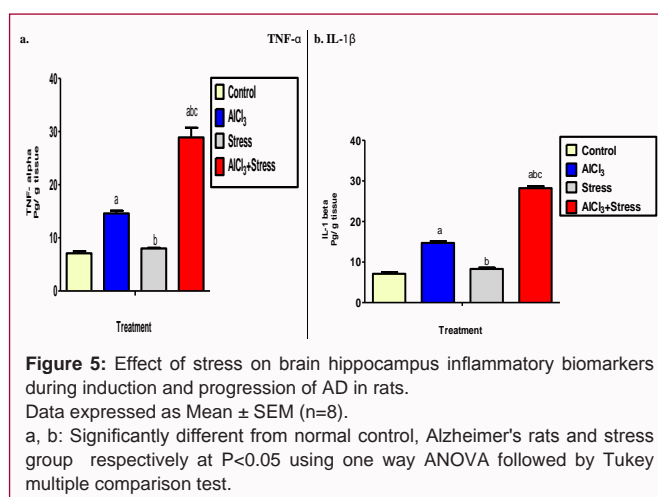
## Materials and Methods

### Animals

The study was conducted in accordance with ethical guidelines of Faculty of Pharmacy, Al-Azhar University, Egypt. Fifty six male Sprague Dawley rats, weighing 220 g-250 g were used. They were

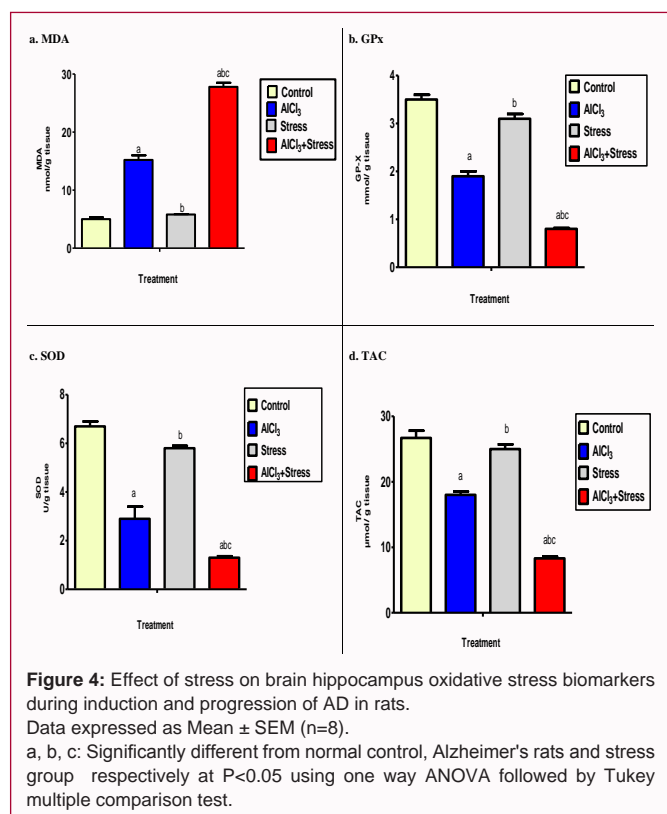


**Figure 3:** Effect of stress on brain hippocampus AChE activity during induction and progression of AD in rats.



**Figure 5:** Effect of stress on brain hippocampus inflammatory biomarkers during induction and progression of AD in rats.

Data expressed as Mean ± SEM (n=8). a, b: Significantly different from normal control, Alzheimer's rats and stress group respectively at P<0.05 using one way ANOVA followed by Tukey multiple comparison test.



**Figure 4:** Effect of stress on brain hippocampus oxidative stress biomarkers during induction and progression of AD in rats.

Data expressed as Mean ± SEM (n=8). a, b, c: Significantly different from normal control, Alzheimer's rats and stress group respectively at P<0.05 using one way ANOVA followed by Tukey multiple comparison test.

obtained from Nile Co. for Pharmaceuticals and Chemical Industries, Cairo, Egypt. Rats were housed in stainless-steel cages, four per cage, with alternatively 12 hour light and dark cycles at a temperature of 25°C ± 1°C. All rats were kept under the same adequate conditions. They provided with their daily dietary requirements of standard diet pellets (El-Nasr, Abu Zaabal, Cairo, Egypt) contained not less than 20% protein, 5% fiber, 3.5% fat, 6.5% ash and vitamin mixture and water was given ad-libitum. One hour before each experiment and after food and water had been removed from the cages; rats were taken to the test situation for adaptation. All experiments were carried during fixed time (from 8 AM to 12 PM).

**Drugs and chemicals**

Aluminum chloride-hydrated (AlCl<sub>3</sub>.6H<sub>2</sub>O), EGCG, and diazepam were purchased from Sigma Chemical Co. (St. Louis, MO, USA). They were freshly dissolved in saline. All other chemicals and solvents were of highest grade-commercially available.

**Experimental Design**

Seven groups (8 rats/group) were daily IP injected for six week

either with saline for control (2 groups) or with 70 mg/kg AlCl<sub>3</sub> [29] for AD-induced model (5 groups). Stress was induced for all groups except one control and one AlCl<sub>3</sub> group by exposing rats 6 times during six weeks to Stress-induced box paradigm (1time/week for 30 minute) [30]. Three groups of AD-induced model were also daily received either EGCG (10 mg/kg, IP) [31], Diazepam (0.1 mg/kg, IP) [30-32] or their combination. All drugs were administered at a dose volume 0.5 ml/250 g body weight. Two behavioral experiments were performed: Morris water maze test and Conditioned-avoidance test. Rats were sacrificed 24 hours after the last test then the brain tissues were dissected and washed with ice-cold saline. All of the brain tissues were either subjected for analysis immediately or kept frozen at -80°C till the time of analysis. Hippocampus tissues were homogenized in saline; the homogenates were used to assess oxidative stress markers as lipid peroxides expressed as Malondialdehyde (MDA), Superoxide Dismutase (SOD), Glutation Peroxidase (GPx) and Total Antioxidant Capacity (TAC) also inflammatory mediators tumor necrosis factor-α (TNF-α) and interleukin- 1β( IL-1β), furthermore, Acetylcholine Esterase (AChE) activity was also estimated.

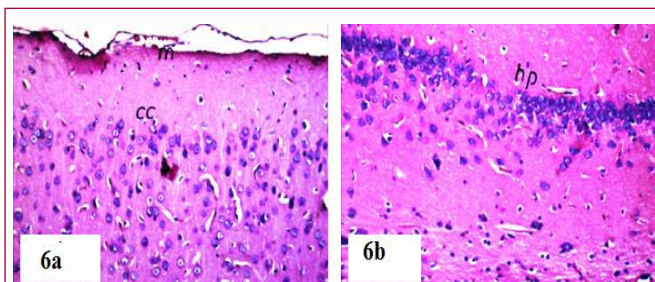
In additions, specimens from the whole brain areas from different treated groups were kept in 10% formaldehyde solution for histopathological examination using Hematoxylin and Eosin (H&E).

**Behavioral Experiments**

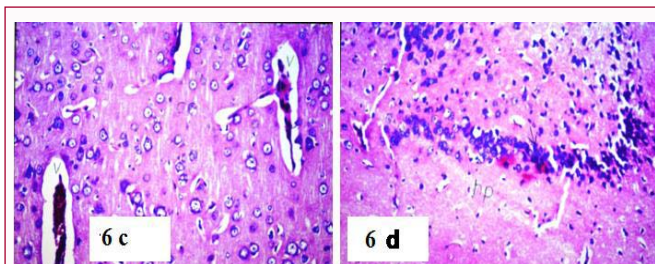
Two experiments of behavioral assessments with different degree of stressfulness were selected to formulate an integrative testing battery. The chosen batteries of tests allow measuring the most behavioral responses to the mentioned drugs. This battery includes the following experiments which were carried out for all the aforementioned groups of animals according to certain sequence depending on their level of stressfulness.

**Morris water maze (MWM) test**

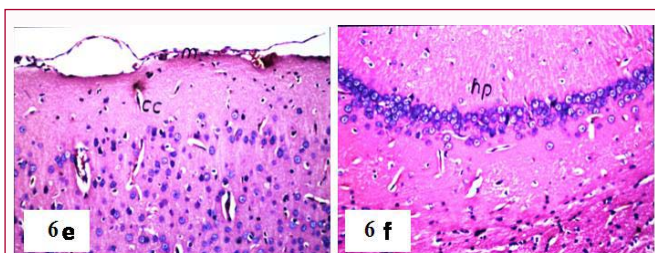
It is considered as hippocampus dependent spatial learning task as previously described [33] in which rats are required to learn to find an escape platform in a pool of water, using visual cues surrounding the maze. Water maze was a black circular tank 150 cm in diameter and 62.5 cm in height. The tank was filled with water (20°C ± 1°C) to a depth of 40 cm. around the room; numerous visual cues (e.g. bookcase and tables) were present which remained constant throughout the experiment. The maze was divided geographically into four quadrants Northeast (NE), Northwest (NW), Southeast



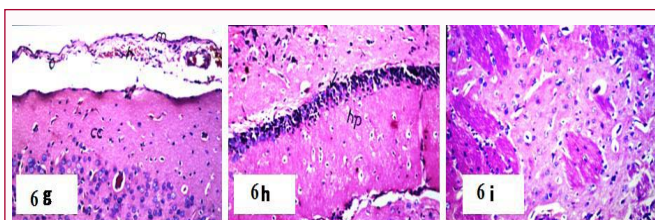
**Figure 6a and b:** Brain of rat in control group showed normal histological structure of the meninges (m), cerebral cortex (cc) (6a) and hippocampus (hp) (6b).



**Figure 6c and d:** Brain of  $AlCl_3$  group showed focal Gliosis (g) and congestion in blood Vessels (v) in Cerebral Cortex (cc) (6c), neuronal pyknosis, degeneration (arrow) and congestion in the blood Vessels (v) in hippocampus (6d).



**Figure 6e and f:** Stressed group showed mild congestion in the blood vessels of the cerebral cortex (6e). There was no histopathological alteration in the hippocampus (6f).



**Figure 6g and i:** Brain of  $AlCl_3$  group exposed to stress showed focal haemorrhage with oedema was detected in the meninges associated with congestion in the blood vessels of the cerebral cortex (6g). Severe neuronal degeneration and pyknosis were noticed in diffuse manner all over the hippocampus cells (6h). Focal eosinophilic plaques formation in striatum (6i).

(SE), Southwest (SW), and starting positions, North (N), South (S), East (E), West (w) that were equally spaced around the perimeter of the pool, a hidden circular platform (diameter: 13 cm) was located in the centre of the SW quadrant, 1 cm below the surface of the water. Rats were trained to find a submerged escape platform, located in a fixed position during four consecutive daily sessions. Each session consisted of four trials; four different starting positions and equally spaced around the perimeter of the pool were used in a fixed order. Each trial had a maximum duration of 60 sec began with releasing the

rats in MWW, then escape latency was calculated which is the time in seconds taken to escape on to the submerged platform. Rats which couldn't find the platform within 60 sec were placed on it. At the end of each trial, rats were allowed to remain on the platform for 20 sec in order to recognize the place well. For all training trials, escape latency was averaged per rat (four different positions), then calculated the averages of the groups. Two hours after the last training trial (the fourth trial of the fourth day), rats were subjected to a memory probe trial during which they swam for 60 sec in the absence of the training platform. All rats started from the same position, opposite to the target quadrant (i.e. the quadrant where the escape platform had been positioned). Time of probe trial was calculated (i.e. time in seconds spent in the target quadrant).

### Conditioned-Avoidance test

All rats were trained in the apparatus of the conditioned avoidance test which was previously described [34-35] and modified [36]. The use of CA test was extended and parameters were manipulated for evaluating learning ability and memory consolidation in high stressful conditions. The apparatus consists of five interconnected chambers; four of them can be electrified using a laboratory DC power supply (Model GPR-6060 D) to deliver the foot shock (Un-conditioned stimulus; 50 volts, 25 pulse /sec) through their stainless steel grid floor. The fifth chamber represents the safety area (Glass floor). Training was conducted by pairing of auditory stimulus (Conditioned stimulus; electric bell) for 5 seconds followed by another 5 seconds of foot shock. Number of trials to avoid the electric shock and reach to safety area during 5 sec of the conditioned stimulus was calculated for each rat at the 1<sup>st</sup> and the 2<sup>nd</sup> day of training which indicating learning ability and short term memory retention.

### Biochemical Parameters

#### Determination of AChE activity

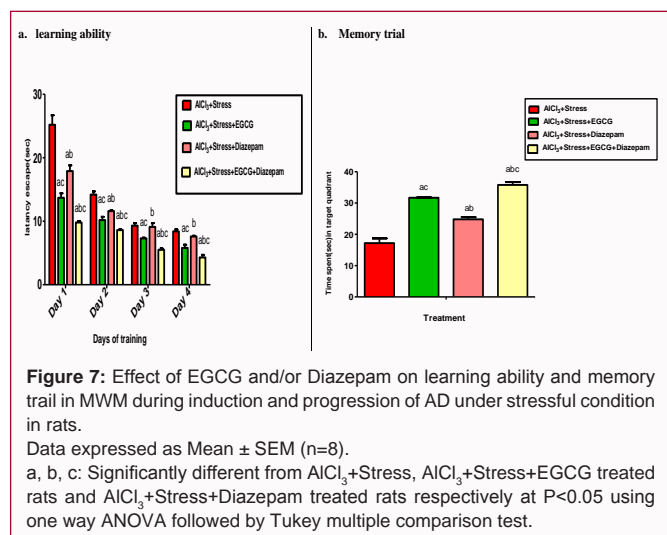
In the brain tissue homogenate, AChE activity was assessed using ELISA Kits (Ray Biotech, Inc., USA) according to the instructions of the manufacturer.

#### Assessment of oxidative stress markers

In all groups, MDA, TAC, SOD and GPx were measured in the brain homogenate of each rat. Lipid peroxidation was determined by estimating the level of Thiobarbituric Acid Reactive Substances (TBARS) measured as MDA [37]. The determination of TAC is performed by the reaction of antioxidants with a defined amount of exogenously provide  $H_2O_2$ . The residual  $H_2O_2$  is determined colorimetrically by an enzymatic reaction which involves the conversion of 3, 5-dichloro- 2-hydroxybenzene sulphonate to a colored product [38]. SOD activity was assessed relying on the ability of the enzyme to inhibit the phenazine methosulphate mediated reduction of nitro blue tetrazolium dye; the increase in absorbance at 560 nm for 5 min is measured [39]. An indirect determination method is used for the analysis of GPx. It is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing Glutathione Reductase (GR) and NADPH (b-Nicotinamide Adenine Dinucleotide Phosphate, Reduced). The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to NADP<sup>+</sup> is indicative of GPx activity, since GPx is the rate limiting factor of the coupled reactions [40].

#### Assessment of inflammatory markers

In the brain tissue homogenate, (TNF- $\alpha$ ) and (IL-1 $\beta$ ) were



assessed using ELISA Kits (Ray Biotech, Inc., USA) according to the instructions of the manufacturer.

### Histopathological Examination for Different Brain Region

Brain specimens were fixed in 10% formalin for 24 hours then washed with tap water; the specimens were prepared and stained for light microscopy [41]. For dehydration, serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used. Specimens were cleared in xylene embedded in paraffin at 56°C in hot air oven for 24 hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by microtome. The obtained tissue sections were collected on glass slides and deparaffinised, they were stained with Hematoxylin and Eosin stain for routine histological examination.

### Statistical Analysis

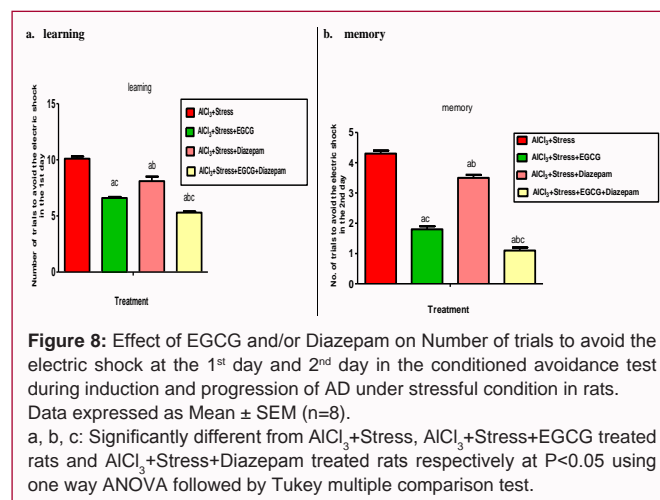
Data was expressed as the mean + SEM and statistical analysis was carried out by one way ANOVA followed by Tukey multiple comparisons test to calculate significance of the difference between treatments. Values of P < 0.05 were considered significant. All statistical analyses were performed and graphs were sketched using Graph Pad Prism (ISI, USA) software (version 5) computer program.

### Results

#### (Part I)

#### Effect of stress on induction and progression of Alzheimer's disease in rats

**Behavioral changes in the MWM:** Efficiency of the learning ability in animals treated with AlCl<sub>3</sub> was decreased. The results in (Figure 1A) showed a significant increase in escape latency from the first to the fourth day of training reaching approximately 214.3%, 187.5%, 133.3% and 1448.8% respectively as compared to control values. Also efficiency of the learning ability in animals subjected to stress and treated with AlCl<sub>3</sub> was decreased. The results in (Figure 1A) showed a significant increase in escape latency from the first to the fourth day of training by approximately 140%, 118.3%, 116.3% and 137.7% respectively as compared to AlCl<sub>3</sub>-treated group. Results in Figure 1B showed that rats treated with AlCl<sub>3</sub> significantly decreased the time spent in the target quadrant reaching approximately 65.5% as compared to the control group. Exposure of the rats treated with AlCl<sub>3</sub> to stress significantly decreased the time spent in target



quadrant by approximately 70.8% as compared to the AlCl<sub>3</sub> treated group.

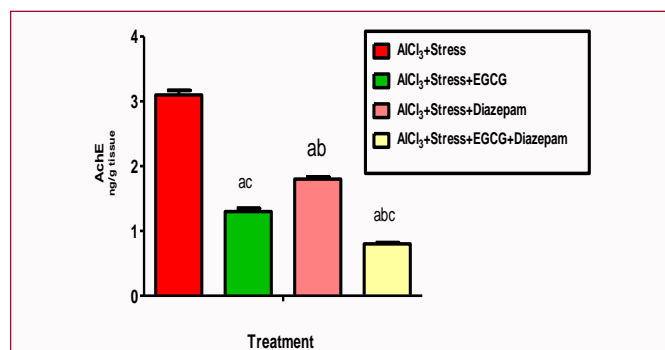
**Behavioral changes in the conditioned - avoidance test:** As shown in Figure 2A, AlCl<sub>3</sub>-treated rats showed marked elevations in the number of trials to avoid the electric shock at the 1<sup>st</sup> day of the experiment amounted to 151.8% with respect to control group. Also AlCl<sub>3</sub>-treated rats exposed to stress showed marked elevation in the number of trials to avoid the electric shock amounted to 118.8% with respect to AlCl<sub>3</sub>-treated rats without stress. In Figure 2B AlCl<sub>3</sub>-treated rats showed marked elevations in the number of trials to avoid the electric shock at the 2<sup>nd</sup> day of the experiment amounted to 216.7% with respect to control group. Also AlCl<sub>3</sub>-treated rats exposed to stress showed marked elevation in the number of trials to avoid the electric shock amounted to 165.4% with respect to AlCl<sub>3</sub>-treated rats without stress.

**Changes in brain acetylcholine esterase (AChE) activity:** Results are shown in Figures 3; AlCl<sub>3</sub>-treated group showed significant increase in the AChE activity to 266.7% as compared to normal control rats. Also AlCl<sub>3</sub>-treated rats exposed to stress showed marked increase in the AChE activity to 193.8% as compared AlCl<sub>3</sub>-treated rats without stress.

**Changes in brain oxidative stress biomarkers (MDA, GPx, SOD and TAC):** Results are shown in (Figure 4A-D); AlCl<sub>3</sub> injection significantly increased MDA level by 304% as compared to the control values. Exposure to stress with AlCl<sub>3</sub> treatment also increased MDA level by 182.9% as compared to AlCl<sub>3</sub> only treated group. While AlCl<sub>3</sub> injection significantly decreased GPx, SOD and TAC levels by 54.3%, 43.3% and 67.4% respectively as compared to the control group. Exposures to stress with AlCl<sub>3</sub> treatment also decreased GPx, SOD and TAC level by 42.1%, 44.8% and 46.1% respectively as compared to AlCl<sub>3</sub> only treated group.

**Changes in brain inflammatory biomarkers (TNF-α, IL-1β):** Results are shown in Figures 5A and B. AlCl<sub>3</sub>-treated group significantly increased level of the TNF-α and IL-1β to 205.6% and 207% respectively as compared to normal control rats. Also AlCl<sub>3</sub>-treated rats exposed to stress showed marked increase in the TNF-α and the IL-1β to 197.9% and 191.8% respectively as compared AlCl<sub>3</sub> treated rats without stress.

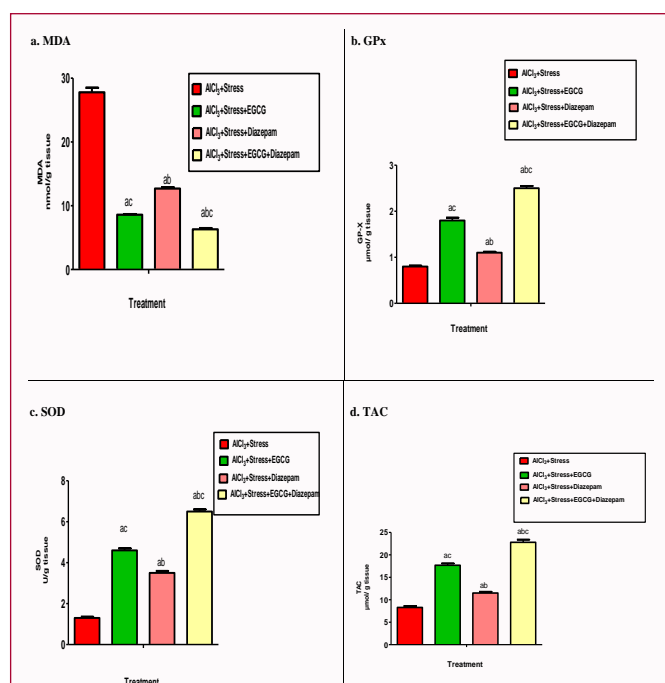
**Histopathological examination of the brain (part I):** There was no histopathological alteration observed in the meninges, cerebral



**Figure 9:** Effect of EGCG and/or Diazepam on brain hippocampus AChE activity during induction and progression of AD under stressful condition in rats:

Data expressed as Mean ± SEM (n=8).

a, b, c, : Significantly different from AICl<sub>3</sub>+Stress, AICl<sub>3</sub>+Stress+EGCG treated rats and AICl<sub>3</sub>+Stress+Diazepam treated rats respectively at P<0.05 using one way ANOVA followed by Tukey multiple comparison test.



**Figure 10:** Effect of EGCG and/or Diazepam on brain hippocampus oxidative stress biomarkers during induction and progression of AD under stressful condition in rats.

Data expressed as Mean ± SEM (n=8).

a, b: Significantly different from AICl<sub>3</sub>+Stress, AICl<sub>3</sub>+Stress+EGCG treated rats and AICl<sub>3</sub>+Stress+Diazepam treated rats respectively at P<0.05 using one way ANOVA followed by Tukey multiple comparison test.

cortex and hippocampus of control rats as recorded in Figure 6A and B. In AICl<sub>3</sub> treated group, focal gliosis was detected in the cerebral cortex and congestion in the blood vessels (Figure 6C), while the hippocampus had pyknosis and degeneration in the neurons (Figure 6D), with congestion in the blood vessels. Stressed group showed mild congestion in the blood vessels of the cerebral cortex (Figure 6 E). There was no histopathological alteration in the hippocampus (Figure 6F). AICl<sub>3</sub> treated rats exposed to stress showed focal haemorrhage with oedema in the meninges associated with congestion in the blood vessels of the cerebral cortex (Figure 6G). Sever neuronal degeneration and pyknosis were noticed in diffuse manner all over the hippocampus cells (Figure 6H) and focal eosinophilic plaques

formation in striatum (Figure 6I). The severity of reaction in brain according to the histopathological alterations can be summarized in Table 1.

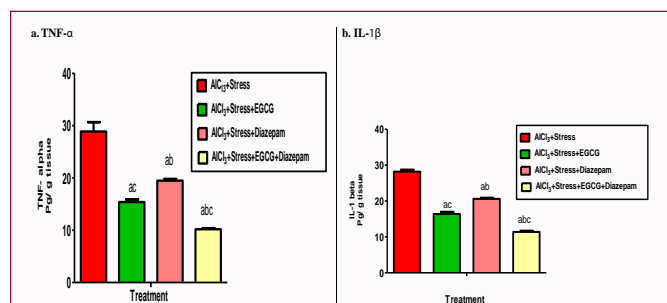
(Part II)

**Effect of Epigallocatechin-3-gallate and/or Diazepam against development of Alzheimer's disease in stressful condition**

**Behavioral changes in the Morris water maze test:** Results are shown in Figure 7A. Efficiency of the learning ability in AD animals exposed to stress and treated with EGCG was increased which indicated by a significant decrease in escape latency from the first to the fourth day of training by approximately 54.4%, 71.8%, 78.5% and 69% respectively as compared to AD group exposed to stress. AD animals exposed to stress treated with diazepam showed decrease in escape latency in first and second day of training by approximately 71%, and 81.7% respectively as compared to AD group exposed to stress. Co-treatment of EGCG and diazepam AD animals exposed to stress decreased in escape latency from the first to the fourth days of training by approximately 38.9%, 60.6%, 59.1% and 51.2% respectively as compared to AD group under stress. Also Co-treatment of EGCG and diazepam showed a significant decrease in escape latency from the first to the fourth day of training by approximately 71.5%, 84.3%, 75.3% and 74.1% respectively as compared to EGCG only treated group and by approximately 54.7%, 74.1%, 60.4% and 56.6% respectively as compared to diazepam only treated group. Results in Figure 7B showed that rats treated with EGCG or diazepam significantly increased the time spent in target quadrant by approximately 184.3% and 144.2% respectively as compared to AD group under stress. Co-treatment of EGCG and diazepam significantly increased the time spent in target quadrant by approximately 208.1% as compared to Alzheimer's group under stress. Also Co-treatment of EGCG and diazepam showed significant increase in the time spent in target quadrant by approximately 112.9% as compared to EGCG only treated group and by approximately 144.4%, as compared to diazepam only treated group.

**Behavioral changes in the conditioned - avoidance test:** As shown in Figure 8A, AD animals exposed to stress and treated with EGCG or diazepam showed marked decrease in the number of trials to avoid the electric shock at the 1<sup>st</sup> day of the experiment amounted to 65.3% , 80.2% respectively with respect to AD group exposed to stress. Co-treatment of EGCG and diazepam significantly decreased in the number of trials to avoid the electric shock at the 1<sup>st</sup> day of the experiment amounted to 52.5% with respect to AD group under stress. However co-treatment of EGCG and diazepam showed significant decrease in the number of trials to avoid the electric shock at the 1<sup>st</sup> day of the experiment amounted to 80.3% with respect to EGCG only treated group and 65.4% with respect to diazepam only treated group.

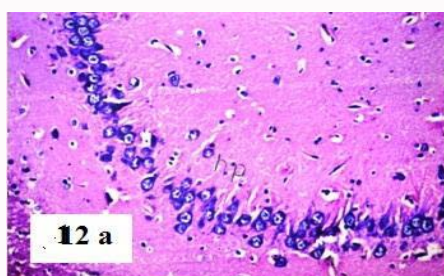
In Figure 8B: AD animals exposed to stress and treated with EGCG or diazepam treated rats showed marked decrease in the number of trials to avoid the electric shock at the 2<sup>nd</sup> day of the experiment amounted to 41.9% and 81.4% respectively with respect to AD group under stress. Co-treatment of EGCG and diazepam significantly decreased in the number of trials to avoid the electric shock at the 2<sup>nd</sup> day of the experiment amounted to 25.6% with respect to AD group under stress. However co-treatment of EGCG and diazepam showed significant decrease in the number of trials to avoid the electric shock at the 2<sup>nd</sup> day of the experiment amounted to 61.1% with respect to



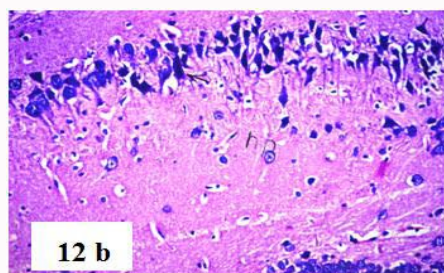
**Figure 11:** Effect of EGCG and/or Diazepam on brain hippocampus inflammatory biomarkers during induction and progression of AD under stressful condition in rats.

Data expressed as Mean ± SEM (n=8).

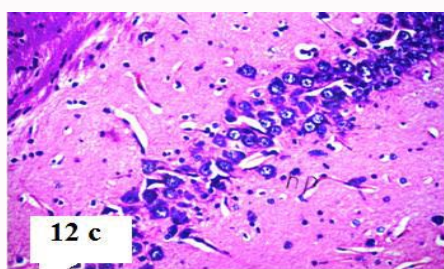
a, b: Significantly different from AICl<sub>3</sub>+Stress, AICl<sub>3</sub>+Stress+EGCG treated rats and AICl<sub>3</sub>+Stress+Diazepam treated rats respectively at P<0.05 using one way ANOVA followed by Tukey multiple comparison test.



**Figure 12a:** Brain of EGCG treated group showed normal histological structure of Hippocampus (HP).



**Figure 12b:** Brain of diazepam treated group showed degeneration in some neuronal cell of hippocampus.



**Figure 12c:** Co-treatment of EGCG and diazepam rats showed normal histological structure of Hippocampus (HP).

EGCG only treated group and 31.4% with respect to diazepam only treated group.

**Changes in brain acetylcholine esterase (AChE) activity:** Results are shown in Figure 9. AD animals exposed to stress and treated with EGCG or diazepam showed significant decrease in the AChE activity to 41.9% and 58.1% respectively as compared to AD group under

stress. Co-treatment of EGCG and diazepam significantly decreased the AChE activity to 25.8% as compared to AD group under stress. However co-treatment of EGCG and diazepam significantly decreased the AChE activity to 61.5% as compared to EGCG only treated group and 44.4% as compared to diazepam only treated group.

**Changes in brain oxidative stress biomarkers (MDA, GPx, SOD and TAC):** Results are shown in (Figure 10 A-D); AD animals exposed to stress and treated with EGCG or diazepam treatment significantly decreased MDA level by 30.9% and 45.7% respectively as compared to AD group under stress. Co-treatment of EGCG and diazepam significantly decreased MDA level to 22.7% as compared to AD group under stress. EGCG treatment to AD animals exposed to stress significantly increased GPx, SOD and TAC level by 225%, 353.8% and 213.3% respectively as compared to AD group under stress. Also AD animals exposed to stress and treated with Diazepam showed significant increase in GPx, SOD and TAC level to 137.5%, 269.2% and 138.6% respectively as compared to AD group under stress. Co-treatment of EGCG and diazepam significantly increased GPx, SOD and TAC level to 312.5%, 500% and 274.7% respectively as compared to AD group under stress.

**Changes in brain inflammatory biomarkers (TNF-α, IL-1β):** Results are shown in Figure 11 A and B. AD animals exposed to stress and treated with EGCG or diazepam treated group showed significant decrease in TNF-α to 53.3% and 67.5% respectively as compared to AD group under stress. Co-treatment of EGCG and diazepam significantly decreased the TNF-α to 35.3% as compared to AD group under stress. Also AD animals exposed to stress and treated with EGCG or diazepam showed significant decrease IL-1β to 58.2% and 73% respectively as compared to AD group under stress. Co-treatment of EGCG and diazepam significantly decreased IL-1β to 40.4% as compared to AD group under stress.

**Histopathological examination of the brain (Part II):** AICl<sub>3</sub> treated rats exposed to stress showed focal haemorrhage with oedema in the meninges associated with congestion in the blood vessels of the cerebral cortex Figure 6G. Sever neuronal degeneration and pyknosis were noticed in diffuse manner all over the hippocampus cells Figure 6H and focal eosinophilic plaques formation in striatum Figure 6I. There was no histopathological alteration in the hippocampus of AD rats exposed to stress treated with EGCG as recorded in Figure 12A. In AD rats exposed to stress treated with diazepam, degeneration with pyknotic nuclei were detected in some of neuronal cells in hippocampus Figure 12B. Co-treatment of EGCG and diazepam to AD rats exposed to stress revealed no histopathological alteration in the hippocampus as recorded in Figure 12C. The severity of reaction in brain according to the histopathological alterations can be summarized in Table 2.

## Discussion

Aluminum (Al) has been suggested as a causal factor in AD, in part because of reports showing the toxicity of Al, the elevation of its concentrations in the brains of patients with AD, and an association between Al concentrations in water and the prevalence of AD [16-17].

The MWM is one of the most common tasks used to assess learning ability and spatial memory in rodents [42]. Results of the current study showed that AICl<sub>3</sub> injection induced a significant increase in escape latency to reach the hidden platform accompanied by a significant decrease in the time spent in target quadrant in MWM

**Table 1:** Histopathological alterations indicating the severity of reaction in brain of rats.

Histopathological Alterations	Control	Stress	AlCl <sub>3</sub> +Stress
Pyknosis & degeneration of hippocampus neurons	-	-	+++
Atrophy in hippocampus	-	-	++
Plaque formation in striatum	-	-	+++
Congestion	-	+	++

+++ Sever ++ Moderate + Mild - Ni

**Table 2:** Histopathological alterations indicating the severity of reaction in brain of rats.

Histopathological Alterations	AlCl <sub>3</sub> +Stress	EGCG	Diazepam	EGCG+ diazepam
Pyknosis & degeneration of hippocampus neurons	+++	-	++	-
Atrophy in hippocampus	++	-	+	-
Plaque formation in striatum	+++	-	-	-
Congestion	++	-	-	-

+++ Sever ++ Moderate + Mild - Nil

which indicates impairment of learning ability and spatial memory. Since Al accumulates in all the regions of the brain maximum being in hippocampus, which is the key site of memory and learning. This result was in agreement with the data reported by [43]. The disruption in memory by AlCl<sub>3</sub> could be attributed to the ability of Al to interfere with downstream effectors molecules, such as cyclic GMP, involved in long-term potentiation [44]; which could then explain the memory impairment and neurobehavioral deficits observed. In addition, in the conditioned-avoidance test, administration of AlCl<sub>3</sub> revealed marked elevation in the number of trials to avoid the electric shock in the 1st and 2nd days of the experiment. These resulting data could be attributed to the deficits in the learning, memory and retrieval abilities (Cognitive functions); the number of trials demonstrated by the animal in the conditioned-avoidance technique is known as a valuable parameter to assess the memory and learning behaviours of the animal [35].

Results of the present study also showed that AD induced by injection of AlCl<sub>3</sub> significantly increased the AChE activity. AChE is a marker of the loss of cholinergic neurons in the forebrain [45]. Functionally Al alters the blood brain barrier and produces changes in the cholinergic and noradrenergic neurotransmission [46]. It is a potent cholinotoxin [47] and has a biphasic effect on AChE activity, with an initial increase in the activity of this enzyme during the first 4-14 days of exposure followed by a marked decrease. This biphasic effect has been attributed to the slow accumulation of Al in the brain [48]. This would explain the increase in AChE activity observed in the AlCl<sub>3</sub> treated rats in the current work. It was previously found that Al has a potent pro-oxidant known to enhance lipid peroxides (MDA) in the cortex and hippocampus [49]. As oxidative damage is mediated by free radicals, it was necessary to investigate the status of endogenous antioxidant enzymes like superoxide dismutase and glutathione peroxidase (GPx), which are the first line of defence against free radical damage under oxidative stress conditions.

In the current work, chronic administration of AlCl<sub>3</sub> resulted in marked oxidative stress as indicated by significant increase in lipid peroxidation (measured as MDA level) and significant decrease in GPx, SOD and TAC. This result is in partial agreement with the data reported by [50] who found that rats treated with Al cause increased MDA levels, decreased SOD levels.

These changes could have been due to the reduced axonal mitochondria turnover, disruption of the golgi or reduction of synaptic vesicles induced by aluminium treatment, all of which result in the release of oxidative products like malondialdehyde, carbonyls, and peroxynitrites within the neurons [51].

Unregulated inflammation and impaired inflammatory control process are highly linked to the pathogenesis of AD. IL-1, with two distinct isoforms of IL-1 $\alpha$  and IL-1 $\beta$ , is considered as one of the most significant cytokines over expressed in the pathogenesis of initial disease [52]. In addition, TNF- $\alpha$  is believed to be the major pro inflammatory response regulator in brain [53]. The cognitive decline in patients can be improved by TNF- $\alpha$  inhibition [54]. This would explain the increase in IL-1 $\beta$  and TNF- $\alpha$  observed in the aluminium chloride treated rats in present work. These results are confirmed by histopathological examination of the brain which showed that chronic administration of AlCl<sub>3</sub> cause focal gliosis which detected in the cerebral cortex and congestion in the blood vessels, while the hippocampus had pyknosis and degeneration in the neurons, with congestion in the blood vessels. These results are in agreement with other work which showed that the volume of the cells has been decreased [55]. In addition, shrinkage and necrosis were observed in scattered cells of hippocampus. Stress alone without AlCl<sub>3</sub> didn't cause any different change compared to control in both behaviour and biochemical parameter. Stress without AlCl<sub>3</sub> didn't cause significant change in escape latency and the time spent in target quadrant in MWM, didn't cause significant change in the number of trials to avoid the electric shock in the 1<sup>st</sup> and 2<sup>nd</sup> days of the experiment in condition Avoidance test. Also stress without AlCl<sub>3</sub> didn't cause any change in MDA, SOD, GPx, TAC, TNF- $\alpha$  and IL1 $\beta$  level. The previously mentioned results regarding stress subjected rats are confirmed by histopathological examination of the brain which showed that stressed group has mild congestion noticed in the blood vessels of the cerebral cortex with no histopathological alteration in the hippocampus.

On the other hand, these results aren't in agreement with the previously reported data which stated that stress affects the hippocampus morphology and that increased corticosterone levels suppress cell proliferation and neurogenesis [56]. Also, rodents under stress can cause cell loss in the CA1 and CA3 hippocampal areas [57]. Neurons in the hippocampal CA1 and CA3 areas are critically important in establishing the correct route during the learning period and then enabling the rats to find the hidden platform in the MWM learning test. CA1 neurons in the hippocampus are active in the acquisition of spatial learning and memory [58]. This discrepancy in result may be due to different stress protocols used.

Results of the current study showed that AlCl<sub>3</sub> treated rat expose to stress (Alzheimer's disease under stressful condition) induced a significant increase in escape latency to reach the hidden platform accompanied by a significant decrease the time spent in target quadrant in MWM which indicate impairment of learning ability and spatial memory more than AlCl<sub>3</sub> only treated rats. In addition, in the conditioned-avoidance test, AlCl<sub>3</sub> treated rats exposed to stress caused marked elevation in the number of trials to avoid the electric shock in the 1<sup>st</sup> and 2<sup>nd</sup> days of the experiment. These resulting data could be attributed to the deficits in the learning, memory and retrieval abilities (Cognitive functions) more than AlCl<sub>3</sub> only treated rats. Results of the present study also showed that AlCl<sub>3</sub> treated rat expose to stress significantly increase the AChE activity ,decrease



antioxidant enzyme (SOD, GPx and TAC) and increase level of MDA and inflammatory marker (IL-1 $\beta$  and TNF- $\alpha$ ) as compared to AlCl<sub>3</sub> only treated rat. The current work is in partial agreement with the data reported by [59] in which application of another form of stress with AlCl<sub>3</sub> showed increased in level of MDA and decreased SOD. The current study also is in agreement with the data reported by [3,5] that stated that stress is believed to contribute to the variability of the aging process and to the development of age-related neuro- and psychopathologies. Clinical data suggest that a stressful lifestyle can be a risk factor for AD [6].

All these results are confirmed by histopathological examination of the brain which showed that administration of AlCl<sub>3</sub> with exposures to stressful condition causes focal haemorrhage with oedema which detected in the meninges associated with congestion in the blood vessels of the cerebral cortex. Severe neuronal degeneration and pyknosis were noticed in diffuse manner all over the hippocampus cells. Focal eosinophilic plaques formation in striatum. Histopathological changes in hippocampus may be explained by the biochemical changes which are previously mentioned.

It is worthy to note that, oxidative stress and cognitive dysfunction are strongly correlated and agents that modulate reactive oxygen species may be potentially useful as anti dementia agents. Co-administration of EGCG and AlCl<sub>3</sub> for six weeks under stressful condition induced a significant decrease in escape latency to reach the hidden platform accompanied by a significant increase in the time spent in target quadrant in MWM as compared to AlCl<sub>3</sub> under stressful condition. This indicates improvement of learning ability and spatial memory. Also, in the conditioned-avoidance test, EGCG treated rat show marked decrease in the number of trials to avoid the electric shock in the 1<sup>st</sup> and 2<sup>nd</sup> days of the experiment as compared to AlCl<sub>3</sub> under stressful condition. These resulting data could be attributed to the improvement in the learning and memory and retrieval abilities (Cognitive functions). These results are in agreement with other data which stated that intraperitoneal administration of EGCG attenuated brain beta-amyloid (A $\beta$ ) neuropathology and improved cognitive function in a transgenic AD mouse model [60]. In particular, EGCG inhibits the fibrillogenesis of A $\beta$  through the binding to the natively unfolded polypeptides and preventing their conversion into toxic aggregates intermediates [61]. Also, it has been reported that the treatment with EGCG in mutant AD mice improved memory function enhancing the  $\alpha$ -secretase function and reducing the activities of  $\beta$ - and  $\gamma$ -secretases with subsequently decrease in the levels of A $\beta$  [62].

The present work revealed that administration of EGCG for six weeks was found to improve not only the memory retention but also reduced oxidative damage and inflammation induced by Al administration and stress. Results of the present study also showed that EGCG-treated rat significantly decrease in the AChE activity, increased antioxidant enzyme (SOD, GPx and TAC) and decrease level of MDA and inflammatory marker (IL-1 $\beta$  and TNF- $\alpha$ ).

The current work was supported by other results in which EGCG increased SOD activity and protected against glycation end products induced neurotoxicity by decreasing ROS and MDA [63].

The results of the present work are in accordance with the data of different studies in which EGCG treatment actually inhibits TNF- $\alpha$  expression and subsequently neuronal damage [64-65]. The present results were confirmed by histological examination of the brain in which chronic administration of EGCG show no histopathological

alteration in the hippocampus. Finally, EGCG is a promising compound which has been proven efficacious in AD animal models and has a wide array of biological effects.

Administration of diazepam with AlCl<sub>3</sub> for six weeks under stressful condition induced a significant decrease in escape latency to reach the hidden platform in first and second day accompanied by a significant increase in the time spent in target quadrant in MWM which indicate improvement of learning ability and spatial memory. Also, in the conditioned-avoidance test, diazepam treated rat show marked decrease in the number of trials to avoid the electric shock in the 1<sup>st</sup> and 2<sup>nd</sup> days of the experiment. These resulting data could be attributed to the improvement in the learning and memory and retrieval abilities (Cognitive functions). A possible explanation for results concerning diazepam effect may be verified studies which state that stress-enabled Long-Term Depression (LTD) may result from hippocampal glucocorticoid receptor activation [66-67] which leads to an increase in glutamate concentration [68-69] and decrease in  $\gamma$ -amino-butyric acid (GABA) concentration in several brain areas including hippocampus [70]. The alteration of glutamate and GABA level in the hippocampus may result in a lower threshold for LTD induction after stress. However, elevates GABA level, and then the threshold for LTD induction may return to physiological level. Moreover, hippocampal LTD has been proposed to play a critical role in spatial learning and memory, especially in memory retrieval [71], stress facilitated hippocampal LTD and subsequently induced a dramatic impairment of spatial memory retrieval. By blocking hippocampal LTD caused by stress with pretreatment of diazepam, the impairment of spatial memory retrieval was fully reversed.

Results of the present study also showed that diazepam treated rat significantly decreased in the AChE activity, increased antioxidant enzyme (SOD, GPx and TAC) and decreased level of MDA and inflammatory marker (IL-1 $\beta$  and TNF- $\alpha$ ). This result in agreement with the study which stated that diazepam decreased level of MDA and increased SOD [72], but the effect of diazepam is less pronounced than that of EGCG. These results are confirmed by histological examination of the brain in which administration of diazepam show degeneration with pyknotic nuclei were detected in some of neuronal cells in hippocampus. Co-treatment of EGCG and diazepam had more pronounced effect on all parameter than each one alone.

## Conclusion and Recommendation

It could be concluded that stress has negative impact on AD and represents a risk factor in induction as well as in progression of the disease. It forms an additional deleterious effect on the brain and exacerbates AD-induced degeneration in the hippocampus as well as impairment of learning and memory. However, the deleterious effect of stress on AD development could be counteracted by co-administration of EGCG and Diazepam. Consequently, antioxidants together with anxiolytic could be recommended as disease modifying agents to decrease the development of AD especially upon exposure to stressful conditions during aging.

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