Possible Involvement of Nitric Oxide Signaling Pathway in the Protective Effect of Clomipramine Against Acute Immobilization Stress-Induced Behavioral and Biochemical Changes in Mice

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Abstract

Frequent and persistent stressful events caused depressive illness. Stress is an aversive stimulus which disturbs physiological homeostasis and reflects a variety of biological systems. The present study was designed to investigate the nitric oxide mechanism in the protective effect of clomipramine against acute immobilization stress-induced behavioral and biochemical alterations in mice.

Methods: Mice were immobilized for 6h. Clomipramine (2.5, 5 and 10mg/kg) were administered 30min before subjecting the animals to acute stress. Behavioral tests (elevated plus maze, mirror chamber, locomotor activity) and biochemical analysis (brain glutamate, malondialdehyde (MDA), nitrite, glutathione (GSH) levels and glutathione peroxidase (GSH-Px) activity) were performed subsequently.

Results: Acute immobilization stress caused anxiety-like behavior, impaired locomotor activity and oxidative stress as compared to naive. Pretreatment with clomipramine in the tested doses significantly reversed immobilization stress-induced behavioral and biochemical alterations. l-arginine (50mg/kg) pretreatment with clomipramine (5mg/kg) significantly attenuated the protective effect of clomipramine. However, l-NAME (10mg/kg) and/or methylene blue (10mg/kg) pretreatment with the same dose of clomipramine significantly potentiated their protective effects which were significant as compared to their effect per se respectively.

Conclusions: Present study highlights the involvement of nitric oxide mechanism in the protective effect of clomipramine against acute immobilization-induced behavioral and biochemical alterations in mice.


Introduction

DEPRESSION is a complex disorder, and the mechanisms underlying its pathogenesis remain unrevealed. Clinical and preclinical evidence indicates that stressful life events and chronic stress are risk factors for developing depression [1]. Stress can be defined as physical and psychological modifications that disrupt the homeostasis and the balance of organisms. It is known as one of the most important reasons of several diseases [2]. Common stress symptoms include irritability, muscular tension, inability to concentrate and a variety of physical reactions, such as headaches, elevated heart rate, blood pressure, etc. Alves de Almeida et al. [3] also reported that both oxidative damage levels and antioxidant defense systems were strongly affected by the different environmental stresses.

Various stress models have been developed to replicate depressive symptoms or identify neurobiological substrates underlying human depression [4]. These models are associated with enhanced free radical generation and altered antioxidant enzyme activities [5]. Immobilization/restraint stress is one of these models that represents an easy and convenient method to induce both psychological (escape reaction) and physical stress (muscle work) resulting in restricted mobility and aggression [6]. The term restraint or immobilization stress involves a specific procedure that limits body movement. Restraint stress stimulates cellular pathways that lead to increase production of free radicals [7]. Repeated exposure to immobilization stress induces long-lasting neuronal hypertrophy in the basolateral amygdala neurons and anxiety-like behavior; meanwhile, induces atrophy and debranching in CA3 neurons of the hippocampus [8].

Nitric oxide (NO) has been implicated in several oxidative pathologies [9]. Nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway is
well known mediator in anxiety like behavior [10]. Vincent and Kimura [11] have shown the presence of NO synthase (NOS) in the area of brain regions involved in anxiety, such as hypothalamus, amygdala and hippocampus. Moreover, number of studies has demonstrated that inhibition of nitric oxide synthase (NOS) produces anxiolytic and antidepressant-like behavioral effects in a variety of animal paradigms [12-13]. L-NAME, an inhibitor of NOS was found to have anxiolytic-like effect [14]. It has also been reported that L-NAME suppress stress-induced enhancement of lipid peroxidation in the brain of mice [15].

Oxidative stress and NO have been proposed to interplay in acute stress pathophysiology, however, exact cellular cascade are not fully understood so far [16]. This encourages researcher to further evaluate new treatment strategy against this disorder. In addition, it was previously found that the NMDA receptor/NO/cyclic GMP pathway can be inhibited by drugs acting through 5-HT receptors. Studies have also shown that inhibition of nitric oxide synthase (NOS) could be used to enhance the clinical efficacy of serotonergic antidepressants [17,18].

Antidepressant drugs are frequently used to manage stress and related problems [19]. In addition, several antidepressants show an inhibitory action on oxidative stress and lipid peroxidation, which increases due to immobilized stress [20]. Furthermore, many studies indicated the involvement of NO in the mechanism of antidepressant and neuroprotective action of many antidepressants in different animal models. Some of these antidepressants include venlafaxine Dhir. et al. [21], imipramine, Krass et al., [22] desipramine, Gaur V, Kumar [23], trazodone Kumar and Garg [24], Fluoxetine, Crespi [25] and bupropion, Dhir A, Kulkarni, [26].

Clomipramine is a well-known tricyclic antidepressant acts by dual inhibition of serotonin and norepinephrine neuronal reuptake. It is used in treatment of major depression, obsessive states, chronic painful conditions, premature ejaculation and enuresis, Patyar et al., [27] while the anxiolytic effect of clomipramine, particularly against acute immobilization stress and the possible involvement of NO in this effect was not sufficiently studied.

Therefore, the present work has been designed to study the anxiolytic effect of clomipramine and to investigate the possible involvement of NO signaling in the mechanism of the protective effects of clomipramine against acute immobilization stress-induced behavior alterations and oxidative stress in mice.

Material and Methods

Clomipramine, l-arginine, l-NAME, methylene blue, reduced glutathione, thiobarbituric acid, serum albumin, Folin-Phenol reagent and Griess reagent were purchased from Fluka Co., (Germany). Ellman's reagent [(5,5-Dithiobis (2-nitrobenzoic acid), DTNB] was purchased from Uptima co. (France). All other chemicals were of analytical grade. Clomipramine, l-arginine, l-NAME and methylene blue solutions were prepared freshly directly before injection by dissolving each in physiological saline and injected intraperitoneally, 30min before the 6-h immobilization stress.

Animals:

Adult male Swiss-Webster mice, weighing 22-25g were obtained from the Animal House of King Saud University were used in all experiments. Animals were maintained at 22-27ºC with free access to water and food ad libitum, under a 12:12 light: Dark cycle (lights on at 7:00h). All experiments were carried out between 9:00 and 16:00h. The experiments were performed in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. The animal-testing protocol used in the present investigation was approved by the Institutional Animal Ethics Committee. All efforts were made to minimize animal suffering.

Experimental protocol:

Animals were divided into eleven groups. Each group consists of 10 animals. Clomipramine (10 and 20mg/kg) was administered intraperitoneally (i.p), 30min before 6 h immobilized stress. L-NAME (10mg/kg, i.p.), l-arginine (50mg/kg, i.p.) and methylene blue (10mg/kg, i.p.) were administered 30min before drug treatment. Animals were immobilized for 6h by taping all the four limbs to board after placing them on their backs using zinc oxide hospital tape. Release was affected by unraveling the tape after moistening with acetone in order to minimize pain or discomfort. In unstressed group, the mice were kept in animal cages. The antianxiety and locomotor activities were assessed after 6h immobilization stress.

Measurement of anxiety:

**Elevated plus maze:**

The test was carried out according to the method described by Henderson et al. [28]. The elevated plus maze consists of four runways (5cm x 30cm)...
arranged in a cross and elevated 37.5 cm above the ground. The runway floors were constructed of textured translucent plastic. Two of the arms are enclosed by 21-cm clear acrylic plastic walls, and two arms are open, except for a slight raised (0.25 cm) edge, which essentially eliminated the problem of mice falling from the open runways. A low-output compact fluorescent bulb, located 1 m above the center of the maze, provided approximately 20 lx illuminance on all runways of the maze. The clear walls allow equal illumination levels on both open and closed runways, thus avoiding a confounding of light aversion and open closed runway preferences. Infrared emitter/detector pairs located around the perimeter of the apparatus detect movement. Beams are positioned to detect both horizontal activity and vertical rearing in each runway and scanning over the edges and ends of open runways. Animals are placed in a clear rectangular bottomless start box in the center of the maze. The start box is lifted and testing begun. The time spent in each arm was recorded, as well as transitions into different arms.

**Mirror chamber test:**

The mirror chamber, designed to detect anxiolytic agents, is based on the principle that many species show approach-avoidance conflict behavior when faced with a mirror image [29]. The outer box containing the chamber is constructed of black plastic, 40 cm x 40 cm x 30.5 cm high. Located within this box is a black 30.5-cm cube, open on one end. The three inner walls, ceiling, and floor of the cube are mirrored. The space between the inner cube and outer box provides the animal with a 4.6-cm dark-walled dim (1-2 lx) alley surrounding the cube. Infrared emitter detectors monitored alley-to-alley transitions, rearing and latency to enter the mirrored chamber. During the 5 min test session, following parameters were noted (a) latency to enter the mirror chamber, (b) total time spent in mirror chamber. Animals were placed individually at the distal corner of the mirror chamber at the beginning of the test. An anxiogenic response was defined as decreased number of entries and time spent in the mirror chamber [30].

**Measurement of locomotor activity:**

Ambulatory movements were recorded by using actophotometer (activity cage, Ugo basile, Italy). The apparatus was placed in a darkened, light sound attenuated and ventilated testing room. Before locomotor task, animals were placed individually in the activity meter for 3 min. The ambulatory movements were recorded for a period of 5 min and expressed in terms of total photo beam counts for 5 min per animal [31].

**Biochemical measurements:**

Animals were sacrificed by decapitation immediately after behavioral assessment. The whole brains were removed, rinsed in isotonic saline and weighed. A 10% (w/v) tissue homogenate was prepared with 0.1 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000 x g for 15 min at 4°C. Aliquots of supernatant were separated and used for biochemical estimations.

**Determination of lipid peroxidation:**

Lipid peroxidation was estimated by the measurement of malondialdehyde (MDA) levels. It is an end product of lipid peroxidation and its level was determined spectrophotometrically by use of thiobarbituric acid reactive substances method previously described by Ohkawa et al., [32].

**Determination of intracellular GSH:**

The GSH content of the neutralized supernatant of hippocampal homogenate was assayed using Ellman’s reagent [5,5-dithiobis-2-nitrobenzoic acid (DTNB solution)] according to the method of Ellman [33].

**Determination of nitrite level:**

Nitrite is the stable product of nitric oxide in living system. Accumulation of nitrite was measured in cell-free supernatants from brain homogenates by spectrophotometer assay based on Greiss reagent 15 (1% sulfanilamide 0.1% naphthyleylenediamine dihydrochloride 2.5% phosphoric acid) and incubated at room temperature for 10 min to yield a chromophore. Absorbance was read at 543 nm spectrophotometrically. The nitrite concentration was calculated from a standard curve using sodium nitrite as standard and expressed as micro molar nitrite per milliliter homogenate [34].

**Determination of GSH-Px activity:**

Glutathione peroxidase activity was measured by the method of Pagila and Valentine [35]. The enzymatic reaction containing β-nicotinamide adenine dinucleotide phosphate (NADPH, GSH, glutathione reductase and a sample or standard was initiated by addition of hydrogen peroxide. The change in the absorbance was measured spectrophotometrically. A standard curve was plotted to calculate the sample concentration. The activity of GSH-Px was expressed as unit/mg protein.

**Determination of protein content:**

The protein content in the supernatant of hippocampal homogenate was measured by method of Lowry et al., [36] with bovine serum albumin.
as the standard.

**Statistical analysis:**
All the values are expressed as mean ± SEM. The data were analyzed by using two-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *p*<0.05 was considered statistically significant.

**Results**

**Effects of clomipramine and its interaction with L-NAME, methylene blue and L-arginine on anxiety like behavior of acute immobilization stressed mice:**

Acute immobilization stress (IS) significantly decreased average time spent per entry in open arm of plus maze (Fig. 1) and delayed latency to enter in mirror chamber (Fig. 2A) and decreased average time spent per entry in the mirror chamber (Fig. 2B) as compared to naïve group (without IS). Treatment with clomipramine (5 and 10mg/kg, ip) significantly increased the average time spent per entry in open arm in plus maze performance task (Fig. 1), shortened the latency to enter mirror chamber (Fig. 2A) as well as increased average time spent per entry in the mirror chamber (Fig. 2B) as compared to control (IS) (*p*<0.05). However, clomipramine (2.5mg/kg) did not produce any significant effect on anxiety as compared to control (*p*<0.05). l-NAME (10mg/kg), the NO synthase inhibitor and methylene blue (10mg/kg) treatment produced antianxiety like behavior in the two test models. However, their effects were not significant as compared to control (IS).

Combination of l-NAME (10mg/kg) and methylene blue (10mg/kg) pretreatment with low effective dose of clomipramine (5mg/kg) caused synergism in their antianxiety effect which was significant as compared to their effect per se (*p*<0.05) (Figs. 1, 2). Further, l-arginine (50mg/kg) did not produce any significant effect on the anxiety like behavior in the two test models as compared to control (*p*<0.05). However, pretreatment of l-arginine (50mg/kg) with clomipramine (5mg/kg) significantly reversed the antianxiety effect of clomipramine (5mg/kg) in the two test models as compared to their effect per se (*p*<0.05) (Figs. 1, 2).

**Effects of clomipramine and its modulation by l-NAME, l-arginine and methylene blue on locomotor activity of acute immobilization stressed mice:**

Exposure of animals to immobilization stress (IS) significantly impaired locomotor activity as compared to naïve mice. Treatment with clomipramine (5 and 10mg/kg, i.p) significantly improved locomotor activity as compared to control (IS) (*p*<0.05). However, clomipramine (2.5mg/kg) did not produce any significant effect on locomotor activity as compared to control (*p*<0.05) (Fig. 3). l-NAME (10mg/kg) and methylene blue (10mg/kg) treatment did not influence significantly locomotor activity as compared to their effect per se (*p*<0.05) (Fig. 3). Combined pretreatment of l-NAME or methylene blue with clomipramine (5mg/kg) significantly improve the locomotor activity of animals as compared to their effect per se (*p*<0.05) (Fig. 3). Although a trend towards significance was observed with both the combinations. Similarly, l-arginine (50mg/kg) and its combination with clomipramine (5mg/kg) did not influence locomotor activity as compared to their effect per se (Fig. 3).

**Effects of clomipramine and its modulation by l-NAME, l-arginine and methylene blue on brain glutamate content of acute immobilization stressed mice:**

Immobilization stress for 6h significantly increased brain glutamate level as compared to naïve mice. Pretreatment of animals with clomipramine (5 & 10mg/kg, i.p) significantly decreased brain glutamate level compared to its level in control (IS) animals. l-NAME (10mg/kg) and methylene blue (10mg/kg) treatment group significantly decreased brain glutamate relative to IS control (Fig. 4). Combination of l-NAME (10mg/kg) and methylene blue (10mg/kg) pretreatment with clomipramine (5mg/kg) caused enhanced in the brain glutamate decreasing activity of clomipramine which was significant as compared to their effect per se (*p*<0.05) (Fig. 4).

l-Arginine, NO precursor in a dose of 50mg/kg did not produce any significant effect on the brain glutamate level as compared to control (IS). However, combination of l-Arginine with clomipramine (5mg/kg) caused a decrease in brain glutamate level that was insignificant compared to clomipramine level but was significant compared to its level with l-Arginine (50mg/kg) (Fig. 4).

**Effects of clomipramine and its modulation by l-NAME, l-arginine and methylene blue on oxidative stress parameters of acute immobilization stressed mice:**

Exposure to immobilization stress for 6h significantly caused oxidative stress as indicated by increased lipid peroxidation, nitrite levels and decreased glutathione level, GSH-Px activity as compared to naïve mice. Treatment with clomi-
pramine (2.5, 5 and 10mg/kg, i.p) significantly restored depleted glutathione, GSH-Px activity as well as attenuated elevated lipid peroxidation and nitrite activity as compared to the control (IS) mice ($p<0.05$) (Table 1).

1-NAME (10mg/kg) and methylene blue (10mg/kg) treatment did not significantly influence oxidative stress parameters as compared to control (IS) ($p<0.05$). However, combination of 1-NAME (10mg/kg) and methylene blue (10mg/kg) pretreatment with clomipramine (5mg/kg) caused an increase in the antioxidant activity of clomipramine which was significant as compared to their effect per se ($p<0.05$) (Table 1).

1-Arginine, as NO precursor in a dose of (50mg/kg) did not produce any significant effect on the oxidative stress indicators as compared to control (IS). Further, 1-arginine (50mg/kg) pretreatment with clomipramine (5mg/kg) significantly reversed the antioxidant activity of clomipramine (5mg/kg) ($p<0.05$) (Table 1).

### Table (1): Effect of clomipramine (2.5, 5 and 10mg/kg, i.p) and its modulation by l-NAME, l-arginine and methylene blue on oxidative parameters of 6-h immobilization stressed mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA (nmol/mg pr)</th>
<th>Nitrite ($\mu$g/ml)</th>
<th>GSH (nmol/mg pr)</th>
<th>GSH-Px (nmol/min/mg pr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>0.118±0.005</td>
<td>162.54±4.9</td>
<td>0.032±0.002</td>
<td>2.65±0.26</td>
</tr>
<tr>
<td>Control (IS)</td>
<td>0.409±0.003</td>
<td>507.5±4.28</td>
<td>0.004±0.0008</td>
<td>507.5±4.28</td>
</tr>
<tr>
<td>CLO (2.5)</td>
<td>0.290±0.005</td>
<td>450.0±3.35</td>
<td>0.010±0.005</td>
<td>450.0±3.35</td>
</tr>
<tr>
<td>CLO (5)</td>
<td>0.222±0.005</td>
<td>373.75±3.57</td>
<td>0.019±0.003</td>
<td>373.75±3.57</td>
</tr>
<tr>
<td>CLO (10)</td>
<td>0.172±0.002</td>
<td>226.25±4.24</td>
<td>0.024±0.002</td>
<td>226.25±4.24</td>
</tr>
<tr>
<td>l-NAME (10)</td>
<td>0.404±0.001</td>
<td>480.52±3.32</td>
<td>0.004±0.001</td>
<td>480.51±3.43</td>
</tr>
<tr>
<td>l-Arg (50)</td>
<td>0.422±0.003</td>
<td>536.25±5.63</td>
<td>0.003±0.001</td>
<td>536.25±5.63</td>
</tr>
<tr>
<td>MB (10)</td>
<td>0.408±0.002</td>
<td>493.27±3.72</td>
<td>0.003±0.002</td>
<td>493.25±3.73</td>
</tr>
<tr>
<td>CLO (5) + l-NAME (10)</td>
<td>0.195±0.002</td>
<td>311.25±4.82</td>
<td>0.023±0.001</td>
<td>311.25±4.82</td>
</tr>
<tr>
<td>CLO (5) + l-Arg (50)</td>
<td>0.347±0.003</td>
<td>444.11±4.81</td>
<td>0.010±0.002</td>
<td>444.11±4.81</td>
</tr>
<tr>
<td>CLO (5) + MB (10)</td>
<td>0.191±0.003</td>
<td>322.5±26.23</td>
<td>0.020±0.001</td>
<td>322.5±26.23</td>
</tr>
</tbody>
</table>

IS = Immobilization stress. 
L-Arg = l-arginine. 
MB = Methylene blue. 
CLO = Clomipramine.

Values are expressed as mean ± S.E.M. 

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Fig. (1): Effect of clomipramine (2.5, 5 and 10mg/kg, i.p) and its interaction with nitric oxide modulators on average time spent per entry (s) in open arm of the plus maze test in 6-h immobilization stressed mice.
Possible Involvement of Nitric Oxide Signaling Pathway in the Protective Effect

Fig. (2): Effect of clomipramine (2.5, 5 and 10 mg/kg, i.p) and its interaction with nitric oxide modulators on (A) Latency to enter mirror chamber (s) (B) Average time spent per entry in mirror chamber (s) of mirror chamber test in 6-h immobilization stressed mice.

Values are expressed as mean ± S.E.M.

\[ \text{L-Arg} = \text{l-arginine.} \]
\[ \text{MB} = \text{Methylene blue.} \]
\[ \text{CLO} = \text{Clomipramine.} \]

\[ *p < 0.05 \text{ as compared to naïve.} \]
\[ b p < 0.05 \text{ as compared to control (AIS).} \]
\[ c p < 0.05 \text{ as compared to clomipramine (5mg/kg).} \]
\[ d p < 0.05 \text{ as compared to l-NAME (10mg/kg).} \]
\[ e p < 0.05 \text{ as compared to MB (10mg/kg).} \]

Fig. (3): Effect of clomipramine (2.5, 5 and 10 mg/kg, i.p) and its interaction with nitric oxide modulators on the locomotor activity of 6-h immobilization stressed mice.

Values are expressed as mean ± S.E.M.

\[ \text{L-Arg} = \text{l-arginine.} \]
\[ \text{MB} = \text{Methylene blue.} \]
\[ \text{CLO} = \text{Clomipramine.} \]

\[ *p < 0.05 \text{ as compared to naïve.} \]
\[ b p < 0.05 \text{ as compared to control (AIS).} \]
\[ c p < 0.05 \text{ as compared to clomipramine (5mg/kg).} \]
\[ d p < 0.05 \text{ as compared to l-NAME (10mg/kg).} \]
\[ e p < 0.05 \text{ as compared to MB (10mg/kg).} \]

Fig. (4): Effect of clomipramine (2.5, 5 and 10 mg/kg, i.p) and its interaction with nitric oxide modulators on brain glutamate concentration of 6-h acute immobilization stressed mice.
Discussion

The present study investigated the possible antianxiety effect of clomipramine in different dose levels against acute immobilization-induced stress (IS) in mice by exposure of the animals to IS for 6h. The antianxiety activity was assessed using elevated plus maze and mirror chamber test. The changes in the antianxiety effect of clomipramine by nitric oxide modulators, l-NAME, l-Argenine and methylene blue were tested. The corresponding changes in brain levels of glutamate, lipid peroxidation, NO and GSH and GSH-Px activity were studied. Results showed that, clomipramine in the tested doses exhibit antianxiety activity against IS in the used two tests of anxiety. This effect was enhanced by concurrent treatment with l-NAME and methylene blue and antagonized by l-Argenine, the fact that reflected the involvement of NO signaling in the antianxiety effect of clomipramine. In addition, this effect was accompanied by decrease in brain glutamate, lipid peroxidation and NO levels that were elevated by IS and increase in brain GSH level and GSH-Px activity that were decreased by IS. These results indicated that modulation of brain glutamate, NO and antioxidant defense contribute in the antianxiety effect of clomipramine.

It was reported that immobilization/restraint stress is an easy and convenient method to induce both psychological (escape reaction) and physical stress (muscle work) resulting in restricted mobility and aggression [37,38]. In addition, the antianxiety activity of clomipramine and its changes by NO modulators was tested using two commonly used tests in the assessment of anxiolytic activity, elevated plus maze test and mirror chamber test [28].

In the present study, 6-h immobilization stress caused significant impairment in locomotor activity, anxiety-like behavior in both of the elevated plus maze test and mirror chamber test, suggesting behavioral alterations in stressed animals. These results are in agreement with previous studies reported that immobilization stress impairs motor activity [39], causes anxiety-like behavior and depression-like behavior Esch et al., [40] in animals. Marked behavioral changes might be due to alteration in the brain regions involved such as hippocampus, amygdala, and prefrontal cortex that undergo structural remodeling that aggravate memory dysfunction, anxiety and aggression [8].

Stress activates hypothalamus-pituitary-adrenal axis (HPA) and alters neurological functions at both central and peripheral level. Acute stress influences significantly behavioral functions and oxidative damage in the discrete areas of the brain and precipitates an anxiety-like syndrome [40,41].

In the present study, clomipramine showed antianxiety-like effect in both of elevated plus maze test and mirror chamber test and improvement in locomotor activity, suggesting its therapeutic potential role against stress-induced behavioral alterations. These effects were not observed with small dose (2.5mg/kg) while were observed with higher doses of clomipramine (5 and 10mg/kg). In addition, these effects were potentiated upon administration of clomipramine in small effective dose (5mg/kg) concurrently with l-NAME, an inhibitor of NOS or methylene blue, an inhibitor of both NOS and soluble guanylyl cyclase while antagonized by l-argenine, the NO precursor. These results indicated that the antianxiety effect of clomipramine is obvious in moderate to large doses and is not obvious in doses 2.5mg/kg or less. Moreover, these results suggest that nitric oxide mechanism could be one of the possible mechanisms in the antianxiety and stress protective action of clomipramine.

Nitric oxide (NO), a free radical and important neuromodulator in the CNS involved in several stress-related disorders including neurodegenerative disorders. It has been demonstrated that restraint stress induces the expression of the inducible NO synthase (iNOS) in rat brain and its inhibition protects against stress-induced cell damage in this model [42].

NOS expression is upregulated in the brain of stressed animals, leading to formation of large amount of nitrogen-reactive species that can lead to neuronal injury [15]. Previous findings demonstrated that NOS inhibitors exert antidepressant-like effects in animal models of depression [43].

In this study, measurement of brain NO levels showed that, exposure of animals to immobilization stress was accompanied by increase in brain NO level relative to its level in the naïve animals. Groups treated with clomipramine showed significant decrease in brain NO level relative to the stress control, in the doses that showed antianxiety activity in the two test models. This effect was enhanced by concurrent treatment of animals by clomipramine with l-NAME or methylene blue and antagonized by concurrent treatment by clomipramine with l-argenine. These findings provided more evident for the involvement of NO mechanism in the effect of clomipramine. Recently, a nitric oxide mechanism has been suggested in the pro-
tective effect of many antidepressant drugs against different models of stress [44,45].

Measurement of brain glutamate level in the present study showed that, exposure of animals to acute immobilization stress elevated markedly the brain glutamate level. This increase accounts the neuronal excitability and anxiety that induced by exposure to immobilization stress. Clomipramine in the doses that showed antianxiety activity decreased the brain glutamate in animals exposed to stress. This effect gradually increased by increasing the clomipramine dose, however this effect was not dose-dependent. The ability of clomipramine to decrease brain glutamate may be seen as an important component in the antianxiety effect of clomipramine. The ability of clomipramine to decrease brain glutamate in this study was potentiated by L-NAME or methylene blue and antagonized by l-arginine, the fact that give an indication about the interrelation between the effect of clomipramine on both NO and glutamate.

It has been reported that glutamate activation of NMDA receptor stimulates calcium influx into cells. Calcium then binds to calmodulin and activates constitutive nitric oxide synthase resulting in stimulating NO formation [46]. Hence, increase in nitric oxide synthase enzyme can lead to NMDA excitotoxicity and initiate the inflammatory cascade and generation of reactive oxygen species. However, it has been found that the activity of inducible nitric oxide synthase (iNOS), a calcium-independent high-output nitric oxide synthase isoform, Ogden and Moor, [47] is induced as a consequence of glutamate release and NMDA receptor activation in rat brain cortex during restraint stress Madrigal et al., [48] and after transient focal cerebral ischemia in rats Perez-Asensio et al., [49]. Moreover, Iravani et al. [50] found that unilateral intrastratial administration of N-methyl-D-aspartic acid to rats produced marked iNOS expression within both astroglial and microglial cells.

In studying the effect of clomipramine on the oxidative stress and changes in antioxidant defenses induced by immobilization stress, results showed elevation of brain lipid peroxidation product, malondialdehyde (MDA) that reflects the damage in neuronal cell membranes induced by reactive oxygen and nitroso (O&NS) radicals released during stress. In addition, there was a decrease in brain GSH level and GSH-Px activity as compared to naive animals. These results are in agreement with previous studies indicated that, immobilization stress is a good model for investigating the alterations occurring in oxidant-antioxidant balance in tissues. Exposure to immobilization stress (3h/day for 15 days) may lead to increment of free radical generation which may have changed antioxidant enzyme activities, and cause protein oxidation and lipid peroxidation of tissues Sahin and Gumuslu, [51].

It is well known that oxidative stress plays a role in the pathogenesis of anxiety and stress [52]. Studies on the underlying mechanisms of stress-induced neuronal damage have demonstrated that corticosterone is released from the adrenal cortex during stress [53] which either induces formation of ROS or decreases antioxidant defense, resulting in an increased neuronal damage in cortical cultures [54]. Oxidative stress can cause cellular damage and neurodegeneration by inducing the reactive oxygen species (ROS) that oxidize vital cellular components such as lipids, proteins and DNA [55]. Oxidative stress arises where there is an imbalance between the production and scavenging of free radicals.

Glutathione and glutathione-related enzymes play a key role in protecting the cells against the damaging effects of reactive oxygen species. Intracellular GSH can act as a reductant, reducing hydrogen peroxide and lipid hydroperoxides directly to H$_2$O, a reaction catalyzed by GSH-Px. Depletion of intracellular GSH, under conditions of continuous intracellular oxidative stress, leads to oxidation and damage of lipids, proteins and DNA by the reactive oxygen species [56].

Thus, depletion of intracellular GSH and decrease in the activity of GSH-Px during immobilization stress, in this study, represents a cause for the increased lipid peroxidation level. Peroxidation of membrane lipids has been implicated as a possible mechanism of oxidative stress-induced lethal injury [57].

Clomipramine pretreatment significantly decreased MDA, nitrite concentrations and restored the depleted reduced GSH, and GSH-Px activity, suggesting its activity to protect against oxidative stress effects and its possible antioxidant properties. Previous studies also support our findings that restrained stress causes robust increase in the production of reactive oxygen species and consequent oxidative stress with a concomitant decline in in vivo antioxidant defense [52]. Moreover, other antidepressants such as imipramine, and venlafaxine Krass et al., [22] reduce the MDA level in restraint stress animals. Pretreatment of L-NAME (10mg/kg) or methylene blue (10mg/kg) with lower
dose of clomipramine (5mg/kg) significantly potentiated their protective effect against oxidative stress and lipid peroxidation as compared to their effect per se. However, l-arginine (50mg/kg, i.p.) significantly reversed the protective effect of clomipramine. These results indicated that, in addition to its effect on glutamate and NO, the antianxiety effect of clomipramine is attributed to its ability to protect against stress-induced oxidative stress and lipid peroxidation in addition to its ability to restore the activity of antioxidant defenses. This effect, according to our results, is affected by NO modulators the fact that may be attributed to the ability of clomipramine to decrease NO production as shown in our results, and consequently inhibition of reactive nitroso radicals formation which by its role exhaust the antioxidant defenses.

Conclusion:

Conclusively, our results demonstrate that clomipramine has the ability to protect against anxiety induced by immobilization-induced stress. The ability of clomipramine to provide this protective effect is positively correlated with its ability to suppress immobilization stress-induced overproduction of NO and glutamate, depletion of intracellular GSH, inhibition of GSH-Px activity and increase of lipid peroxidation level. Thus, the protective effect of clomipramine against immobilization stress-induced anxiety may due to inhibition of NO overproduction and maintenance of intracellular antioxidant status.

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