

## Effects of thymoquinone in a rat model of reserpine-induced depression

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The objective of this study is to examine the antidepressant and antioxidant effects of thymoquinone (TQ) on reserpine-induced depression, and to investigate the antidepressant and antioxidant activity of combined treatment of TQ+citalopram. In total, 36 male Wistar rats were randomly divided into 6 groups: 1)control1, 2)control2, 3)reserpine, 4)reserpine+TQ 5)reserpine+citalopram and 6)reserpine+TQ+citalopram. Depression was induced by administering intraperitoneal reserpine of 0.2mg/kg/14days. For antidepressant effects, 10 mg/kg TQ and/or 10 mg/kg citalopram was administered intragastrically 30 minutes prior to the administration of reserpine. Rat behavior was examined using the Behavioral Test following the completion of treatment protocol. Total nitric oxide (NOx) levels, malondialdehyde (MDA) levels, total oxidants status (TOS), total antioxidant status (TAS) in brain cortex, plasma as well as brain cortex glutathione (GSH) and levels of plasma total sulfhydryl groups (RSH) were examined. Treatment with TQ ameliorated the reserpine-induced changes in the Behavioral Test ( $p<0.05$ ). TQ treatment significantly increased dopamine (DA) and noradrenaline (NA) expressions when compared to the R group ( $p<0.01$ ). Serotonin (5-HT) expression also increased significantly ( $p<0.05$ ). Brain cortex and plasma TOS, MDA and NOx levels decreased, whereas TAS, GSH and RSH levels increased ( $p<0.05$ ). TQ has the ability to prevent depression induced by reserpine. The combination of TQ+citalopram can be used in the treatment of depression with a stronger antioxidant effect

**Keywords:** Depression. Thymoquinone. Reserpine. Oxidative stress. Behavioral test.

### INTRODUCTION

Depression is a widespread mental disorder affecting more than 15% of the population throughout their lives (Richards, 2011). The World Health Organization predicts that depression will be the second leading cause of the loss of human disability-adjusted life year worldwide (Rojas *et al.*, 2011). Monoamine neurotransmitters in central nervous system such as dopamine (DA), noradrenaline (NA) and serotonin (5-HT) monoamine play a key role in the pathophysiology of depression (AS, 2004). However, oxidative stress caused by reactive oxygen substances (ROS) can be one of the main reasons behind this disorder. It leads to the destruction and autoxidation of

endogenously produced ROS monoamines in cytosol (Miller *et al.*, 1999).

Reserpine blocks the reuptake of vesicular monoamine and causes the depletion of amines in the brain. Therefore, it is used in the induction of depression in laboratory animals and can be preferred in the assessment of antidepressant activity experimentally (Nagakura *et al.*, 2009). Many antidepressant therapies targeting monoamines such as tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), noradrenaline reuptake inhibitors (NRIs), serotonin and noradrenaline reuptake inhibitors (SNRIs) and monoamine oxidase inhibitors (MAOIs) have been developed based on this theory (Torres, Gainetdinov, Caron, 2003). Selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed first-line antidepressant drugs in depression treatment nowadays. Citalopram is a potent and selective inhibitor of 5-HT reuptake (Jiang, Davidson,

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2005). In extended use, 5-HT, NA and DA cause reuptake (Feng *et al.*, 2018). Due to unfavorable side effects of some commonly used chemical drugs, there is an increasing trend in the public to use herbal medicines to treat various diseases. Herbal medicine has been widely used for the treatment of mood and anxiety disorders since the ancient times. Nowadays, about 25% of all prescribed drugs are derived from herbs or medicinal plants. Many medicinal plants are recently identified for the treatment of specific disorders, but some others have been used for thousands of years without being recognized by scientists (Saki, Bahmani, Rafieian-Kopaei, 2014).

Thymoquinone (TQ) is a bioactive molecule obtained from the plant, *Nigella Sativa* also known as black cumin that grows in Mediterranean region. TQ has antioxidant, anti-inflammatory, and neuroprotective effects. It especially deactivates free oxygen radicals such as hydroxyl radicals and superoxide anions. TQ inhibits lipid peroxidation of cell membrane.

Long term empirical research on TQ carried out on rats found no toxic effects and TQ was reported to have a wide safety range (Sagit *et al.*, 2013).

In a study performed on diabetic rats, it was reported that the use of TQ, due to its antiinflammatory and antioxidants effects, in combination with fluoxetine may be useful in the amelioration of depression aggravated by diabetes (Safhi *et al.*, 2019).

In another study, it was suggested that TQ might have antidepressant effects by decreasing Indoleamine-2,3-dioxygenase activation, causing an increase in hippocampal 5-HT levels and preventing inflammation (Alam *et al.*, 2020).

In literature, there are also studies suggesting that TQ, with its antiinflammatory and antioxidant effects, has ameliorating effects on lipopolysaccharide-induced learning and memory deficits, and that it prevents learning and memory deficits in cases of hypothyroid juvenile rats and cerebrovascular insufficiency and dementia (Fanoudi *et al.*, 2019, Baghcheghi *et al.*, 2018, Bargi *et al.*, 2017).

However, in literature, there are few studies on the effects of TQ on depression. Therefore, an examination of the advantages of TQ whose neuroprotective and antioxidant effects have been studied over conventional antidepressants would be interesting. Our objective in this

study is to examine the antidepressant and antioxidant effects of TQ on reserpine-induced depression and to investigate the antidepressant and antioxidant activity of combined treatment of TQ+citalopram.

## MATERIAL AND METHODS

### Animals

For the study, adult male Wistar Albino rats (weighing 250±20 g) were attained from the laboratory animal center. Rats were maintained in a standard 12 h light/dark cycle in cages with free access to food and water and were allowed to acclimate to the environment for 14 days. Approval for each experimental procedure was obtained from the Ethics Committee on Animal Care and Use (Project number G.Ü.ET-16.012). Every effort was made to ensure minimal suffering of animals.

### Drugs and chemicals

Reserpine, TQ (CAYMAN Chemical, USA), Citalopram hydrobromide (Sigma-Aldrich, USA), Tween 80 (Biomatic, USA), sucrose (Wisent Bioproducts, Canada), TAS and TOS (YL BİONT, China), anti-Noradrenalin (Abcam ab8887, UK), anti-Dopamin Receptor R1 (Abcam ab20066, UK) and anti-Serotonin (AbD Serotec, Thailand) were used in the present study.

### Induction of depression

Depression was induced in rats by intraperitoneal (i.p) injection of freshly prepared reserpine (0.2 mg/kg b.w) dissolved in %1 Tween 80 once daily for 14 days.

We analyzed reserpine-induced depressive disorder through a variety of behavioral and biochemical tests. In order to evaluate the duration of the obtained reserpine effect, the tests were carried out 120 min after the last injection (Antkiewicz-Michaluk *et al.*, 2014).

### Experimental design

In this experiment, a total of 36 rats (24 depressive rats, 12 normal rats) were used. The rats were divided

into six groups as six animals in each group. They were treated for 14 consecutive days as follows: (Table I)

C1 and C2 groups were administered only solvents of the drugs. Reserpine was injected intraperitoneally 0,2 mg/kg once daily. TQ (10 mg/kg) and citalopram (10 mg/kg) were dissolved in tap water and administered

intragastrically 30 min before each reserpine injection. In R+C+T group, citalopram and TQ were administered consecutively following reserpine injection. The drugs were prepared freshly each day and injected in a volume of 1 ml/kg. Administration was conducted from 8:00 to 10:00.

**TABLE I** - Experimental design

Group	Number of rats	Explanations
C 1	n=6	Received i.p. %1 Tween 80
C 2	n=6	Received i.p. %1 Tween 80 and intragastrically tap water
R	n=6	Received Reserpine
R+C	n=6	Received Reserpine and Citalopram
R+T	n=6	Received Reserpine and TQ
R+C+T	n=6	Received Reserpine and Citalopram and TQ

**C1:**Control 1, **C2:** Control 2, **R:** Reserpine, **R+C:** Reserpine+Citalopram, **R+T:** Reserpine+TQ, **R+C+T:** Reserpine+Citalopram+TQ

## Behavioral Studies

### Forced Swimming Test (FST)

In order to evaluate depression severity, we performed a modified forced swimming test (FST), according to the previously described method (Ji *et al.*, 2017).

A pre-test was applied to rats for 15 min to remove the acute stress by water and to adapt them to water. After twenty-hour pre-test, the rats were tested for 5 min. The animals were individually put into a glass cylinder of 15 cm in diameter and 50 cm in height, filled with water to a height of 30 cm. Water temperature was set to 25°C±1°C. Throughout the test session, immobility time, climbing time and swimming time was recorded by a video camera located above the cylinder for the next analysis (Schiavone *et al.*, 2017).

*Immobility:* total absence of active movements apart from minor efforts to keep the head afloat;

*Swimming:* active swimming of the animal pedaling and moving around the cylinder with all four paws absorbed in water;

*Climbing:* dynamic efforts to climb the walls of the cylinder with the animal floating upright extending its front paws.

### Tail Suspension Test (TST)

Rats were individually hung by the tail from a horizontal bar (75 cm above the table top) by a tape affixed 1 cm below the adhesive tape on the tail, in an attempt to avoid tail climbing. Immobility duration of rats were separately recorded for 5 minutes and evaluated. Behaviors of the rats were recorded by a video camera, and analyzed to identify parameters of total immobility duration and an average duration of an immobility episode (the ratio of total duration of immobility and the number of immobility episodes) (Gupte, Dawane, Wele, 2016).

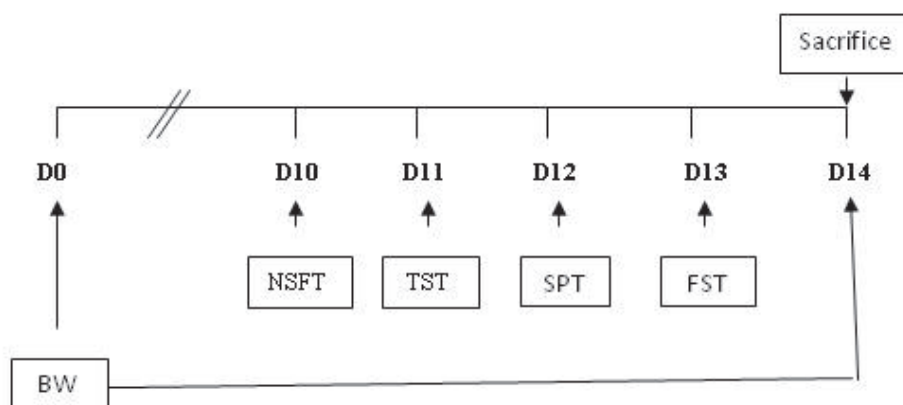
### Novelty suppressed feeding test (NSFT)

The rats were transferred to the testing room, placed in a clean holding cage, and allowed to habituate for 30 min. On the test day, the food-deprived rats (18 h) were individually placed in an open field (60 cm × 40 cm) with a small amount of food in the center of the box on white circular filter paper (10 cm in diameter) at a consistent corner of the chamber. The behaviors were videotaped. Immediately afterwards, the animal was transferred to its home cage. The amount of time to take the first bite was recorded as the latency to feed and the amount of food consumed by the rat in the subsequent 5 min was measured. The NSF arena was thoroughly cleaned with 70% EtOH between animals (Mahmoud *et al.*, 2016).

### Sucrose preference test (SPT)

Sucrose preference test was based on a previous research (Sadeghi, Peeri, Hosseini, 2016). Two bottles of tap water were placed in the home cage of each rat on the first two days. During the second two days, one of the bottles was replaced with a bottle containing 1% sucrose solution. On the test day, animals were not given food and water for 8 hr and sucrose preference was measured during one hour of liquid consumption by utilizing two bottles of 1% sucrose solution and tap water. SPT was assessed using the following equation that calculate the ratio of 1% sucrose solution consumed in the total liquid consumed: Sucrose preference = Sucrose consumed / (Sucrose consumed + tap water consumed).

The timeline for behavioral tests is presented in Figure 1.



**FIGURE 1** - Timeline depicting the sequence of experimental events. D indicates day of experiment. Rats were tested in the novelty suppressed feeding test (NSFT) on day 10, in the tail suspension test (TST) on day 11, in the sucrose preference test (SPT) on day 12 and in the forced swim test (FST) on day 13. Body weight (BW) was taken on day 0 and day 14.

## Biochemical Analyses

### Tissue Preparation

After the behavioral tests, the rats were sacrificed by taking blood from their hearts under anesthesia with intra-muscular (IM) rompun (5mg / kg) + ketamine (45mg / kg). After decapitation, coronal sections

including nuc. accumbens and hypothalamus regions with a thickness of 2-4 mm were immediately taken from the brain tissue and these sections were kept in 4% buffered paraformaldehyde for histopathologic and immunopathologic examination. The remaining brain cortex region and separated plasma removed from rats were frozen in liquid nitrogen and stored at -80°C until biochemical studies.

*Immunohistochemistry for DA, NA and 5-HT expressions*

Immunohistochemistry was performed for the detection of the DA-positive, NA-positive and 5-HT-positive cells in the nuc. accumbens and hypothalamus, as a previously described method (Vacher *et al.*, 2002). All immunoperoxidase analyses were performed using a commercial immunoperoxidase kit (Thermo Fisher Scientific, Massachusetts, USA) and in compliance with the directions given in the kit. For negative control purposes, non-immunized normal rat serum was used instead of primary antibody in each test. For immunoperoxidase staining, briefly the following method was followed: The sections were deparaffinized in xylol series for 5 min each and were rehydrated by being kept in graded alcohol series for 5 min each. For antigen retrieval, the tissues that were boiled in citrate solution (pH 6.0) for 30 min were treated in 1% hydrogenperoxide for 15 min and thus, endogenous peroxidase activity was inhibited. Then, the sections were incubated with protein blocking serum (Thermo Fisher Scientific, Massachusetts, USA) for 10 min. The sections were then incubated with primary antibodies (anti-Noradrenalin, Abcam ab8887; anti-Dopamin Receptor R1, Abcam ab20066 and anti-Serotonin, AbD Serotec) at room temperature for 1 hour, and with secondary antibodies and streptavidin-peroxidase enzyme for 30 min, respectively. The sections that were washed with PBS were then stained with AEC chromogen and Mayer's hematoxylin and closed with water-based adhesive. Stains were assessed using Olympus BX51 (Japan) microscope with a DP25 camera add-on and their microphotographs were taken.

*Histomorphometric Analyses*

Following immunoperoxidase staining, 3 microphotographs of each case were taken with a magnification of 10x using a Leica DM 5000B microscope with a trinocular digital camera add-on. Then, utilizing Leica Q win histomorphometric analysis program, immunopositive stained regions were marked and analysed automatically.

*Determination of plasma and tissue lipid peroxide level*

For the quantification of lipid peroxidation, the formation of thiobarbituric acid reactive substances was measured as described previously (Kustimur *et al.*, 2007).

The supernatants were added into 1 ml of a solution with 15% (wt/vol) tricarboxylic acid, 0.375% (wt/vol) thiobarbituric acid, and 0.25 N HCL following the centrifugation of aliquots (0.5 ml). Protein precipitate was eliminated through centrifugation and the supernatants were placed in glass test tubes with 0.02% (wt/vol) butylated hydroxytoluene with the aim of avoiding further peroxidation of lipids in the preceding steps. Next, the samples were heated at 100°C in a boiling water bath for 15 min, cooled, and centrifuged to eliminate the precipitant. The absorbance of each sample was decided at 532 nm. The expression of lipid peroxide levels was achieved with regards to MDA equivalents by employing an extinction coefficient of  $1.56 \cdot 10^5 \text{ mol}^{-1}$ . Tissue samples were homogenized in ice-cold trichloroacetic acid (1 g tissue plus 10 ml 10% trichloroacetic acid) in a blender with equal volume of 0.67% TBA and heated to 100°C for 15 min. Next, the absorbance of the samples were spectrophotometrically calculated at 535 nm. The lower limit of detection of TBARS (thiobarbituric acid reactive substances) is 0.03  $\mu\text{mol/l}$ .

*Determination of plasma RSH level and tissue GSH levels*

The RSH levels were assessed with a previously presented method (Kustimur *et al.*, 2007). 0.5 ml of each sample was blended with 1 ml of a solution with 100 mM Tris-HCl (pH 8.2), 1% sodium dodecyl sulfate, and 2 mM EDTA. Next, the mixture was incubated for 5 min at 25°C and centrifuged to eliminate any precipitant. 5,5-dithiobis (2-nitrobenzoic acid)/DTNB 0.3 mM was added to each reaction volume and incubated for 15 min at 37°C. The absorbance of each sample was 412 nm. The GSH levels were calculated by a previously described method (Kustimur *et al.*, 2007). In brief, following the centrifugation at 3,000g for 10 min, 0.5 ml of supernatant was added to the 2 ml of dithiobisnitrobenzoate (0.4



mg/ml 1% sodium citrate) and the absorbance at 412 nm was immediately calculated after mixing. The RSH levels were measured with the estimation of a molar extinction coefficient of 13,000 at 412 nm. The GSH levels were measured with an extinction coefficient of 13,600 mol<sup>-1</sup> cm<sup>-1</sup>. The lower limit of detection of GSH was 0.5 µmol/l.

#### *Determination of plasma and tissue total nitric oxide levels*

To attain plasma and brain NO<sub>x</sub> levels from ELISA reader, Vanadium chloride (VCl<sub>3</sub>)/Griess assay was employed (Miranda, Espey, Wink, 2001). Tissues were homogenized in five volumes of phosphate buffer saline (pH = 7) prior to the determination of NO<sub>x</sub>, and then centrifuged at 2000 × g for 5 min. Following centrifugation, 0.25 ml of 0.3 M NaOH was added to 0.5 ml supernatant. The samples were incubated for 5 min at room temperature and 0.25 ml of 5% (w/v) ZnSO<sub>4</sub> was added for deproteinization. Next, the obtained mixture was centrifuged at 3000 × g for 20 min and supernatants were employed for the assays. Nitrate standard solution was consecutively diluted and the plates were loaded with samples (100 µl). Then, Vanadium III chloride (VCl<sub>3</sub>) (100 µl) and Griess reagents sulphanilamide (SULF) (50 µl) and N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) (50 µl) were included in each well. Following the incubation of samples at 37°C for 45 min, they were assessed at 540 nm with ELISA reader. The NO (nitrite + nitrate) levels were estimated with the method proposed by Miranda, Espey and Wink, (2001). Following the centrifugation of blood samples, the supernatants were divided. Samples were deproteinised with 0.3 M NaOH and 5% (w/v) ZnSO<sub>4</sub>, centrifuged at 14 000 rpm for 5 min, and supernatants were utilized for the assays. Experiments were carried out at room temperature. Nitrate standard solution was consecutively diluted. After loading the plate with samples (100 µl), vanadium III chloride (VCl<sub>3</sub>) (100 µl) was added to each well and this was quickly followed by addition of Griess reagents, sulphanilamide (SULF) (50 µl) and N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) (50 µl). After incubation (usually 30-45 min), samples were measured at 540 nm by ELISA reader.

#### *Determination of plasma and tissue Total Antioxidant Status (TAS) and Total Oxidant Status (TOS)*

Blood plasma: EDTA is added, then mixed for 10-20 minutes and centrifuged (at 2000-3000 RPM) for approximately 20 minutes. The supernatants are attentively gathered and kept at -80°C until use. Tissue sample: The sample is incised and weighed up. A certain amount of PBS (PH 7.4) is added and rapidly frozen with liquid nitrogen for later use. The sample is thawed and stored at 2-8°C. A certain amount of PBS (PH 7.4) is added, then the sample is thoroughly homogenized by hand or homogenizer, and centrifuged (at 2000-3000 RPM) for nearly 20 minutes. The supernatants are attentively gathered and kept at -80°C until use. The plasma and brain cortex level of TAS and TOS was measured with ELISA kits.

#### **Statistical analysis**

All data are expressed as the mean ± standard deviation (SD). Data were analyzed by using Statistical Package for Social Sciences 15.0 software program. Comparisons among groups were performed using one-way analysis of variance, followed by *post hoc* Tukey tests. Paired Samples T test was used for the evaluation of body weight. Kruskal Wallis variance analysis was used for the evaluation of tail suspension test. *p* < 0.05 is considered statistically significant.

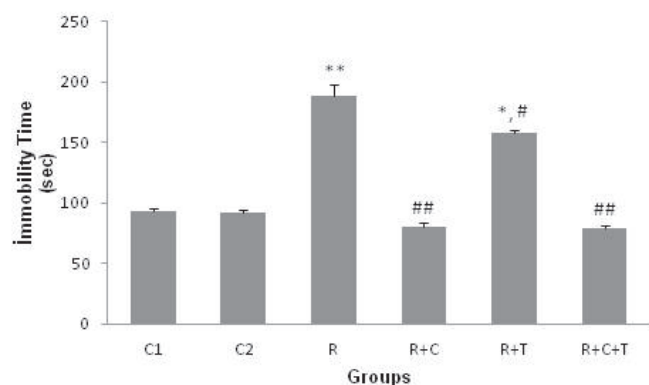
## **RESULTS**

### **The Effect of Reserpine on the Behavioral Tests Carried out 120 min After the Last Injection**

#### *Forced swimming test (FST)*

Compared to the C1 group, the depressed rats exhibited a typical depressive-like behavior after 14 days of reserpine injection such as less climbing, less swimming and extended immobility in FST (*p* < 0.01). Citalopram and citalopram+TQ treatment induced a significant reduction in immobility time and an increase in climbing time and swimming time in FST compared to the rats receiving only reserpine (*p* < 0.01).

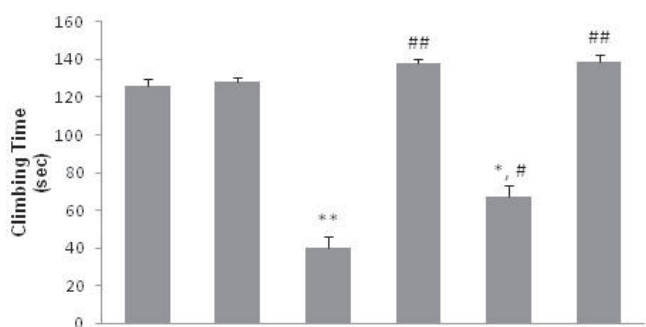
The climbing time, immobility time and swimming time in treatment with citalopram and citalopram+TQ was statistically similar ( $p > 0.05$ ). Compared to the R group, TQ treatment reduced immobility time but increased climbing and swimming time ( $p < 0.05$ ) (climbing time:  $F(5, 30) = 527.096$ ; immobility time  $F(5, 30) = 62.808$ ; swimming time:  $F(5, 30) = 41.896$ ). (Figure 2 A, B, C)



**FIGURE 2A - Immobility time (sec)** The values are means $\pm$ SD;  $n = 6$ .

\* $< 0.05$  versus C2 groups, \*\* $p < 0.01$  versus C1 groups, # $< 0.05$  versus R group, ## $< 0.01$  versus R group

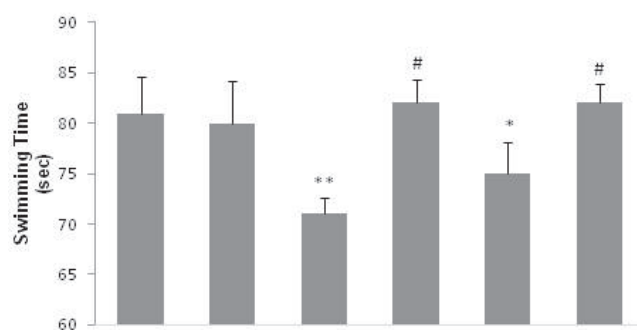
**C1:** Received i.p. %1 Tween 80, **C2:** Received i.p. %1 Tween 80 and intragastrically tap water, **R:** Received Reserpine, **R+C:** Received Reserpine and Citalopram, **R+T:** Received Reserpine and TQ, **R+C+T:** Received Reserpine and Citalopram and TQ



**FIGURE 2B - Climbing time (sec)** The values are means $\pm$ SD;  $n = 6$ .

\* $< 0.05$  versus C2 groups, \*\* $p < 0.01$  versus C1 groups, # $< 0.05$  versus R group, ## $< 0.01$  versus R group

**C1:** Received i.p. %1 Tween 80, **C2:** Received i.p. %1 Tween 80 and intragastrically tap water, **R:** Received Reserpine, **R+C:** Received Reserpine and Citalopram, **R+T:** Received Reserpine and TQ, **R+C+T:** Received Reserpine and Citalopram and TQ



**FIGURE 2C - Swimming time (sec)** The values are means $\pm$ SD;  $n = 6$ .

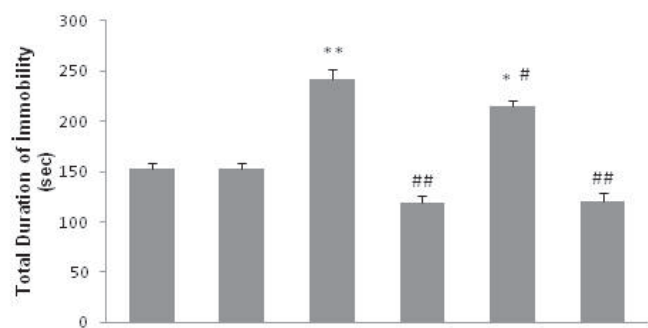
\* $< 0.05$  versus C1 and C2 groups, \*\* $p < 0.01$  versus C1 groups, # $< 0.05$  versus R group

**C1:** Received i.p. %1 Tween 80, **C2:** Received i.p. %1 Tween 80 and intragastrically tap water, **R:** Received Reserpine, **R+C:** Received Reserpine and Citalopram, **R+T:** Received Reserpine and TQ, **R+C+T:** Received Reserpine and Citalopram and TQ

#### Tail Suspension Test

##### Tail suspension test (TST)

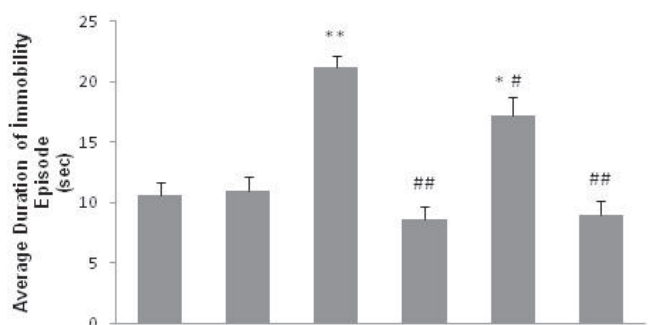
There was an increase in the total duration of immobility and average duration of immobility episode in the R group when compared to the C1 group ( $p < 0.01$ ). The total duration of immobility and the average duration of immobility episode significantly decreased in the citalopram and citalopram+TQ treatment groups compared to the R group ( $p < 0.01$ ). But TQ treatment caused a lower reduction compared to the R group in the total duration of immobility and the average duration of an immobility episode ( $p < 0.05$ ). There was no statistical difference in citalopram treatment group and citalopram + TQ treatment group ( $p > 0.05$ ) (The total duration of immobility:  $t(5) = 32.675$ ; Average duration of immobility episode:  $t(5) = 30.257$ ). (Figure 3 A, B)



**FIGURE 3A - Total Duration of Immobility (sec)** The values are means±SD; n = 6.

\* < 0.05 versus C1 and C2 groups, \*\*p < 0.01 versus C1 groups, # < 0.05 versus R group, ## < 0.01 versus R group

**C1:** Received i.p. %1 Tween 80, **C2:** Received i.p. %1 Tween 80 and intragastrically tap water, **R:** Received Reserpine, **R+C:** Received Reserpine and Citalopram, **R+T:** Received Reserpine and TQ, **R+C+T:** Received Reserpine and Citalopram and TQ



**FIGURE 3B - Average Duration of Immobility Episode (sec)** The values are means±SD; n = 6.

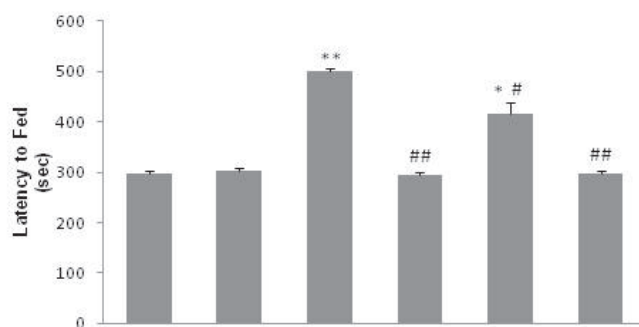
\* < 0.05 versus C1 and C2 groups, \*\*p < 0.01 versus C1 groups, # < 0.05 versus R group, ## < 0.01 versus R group

**C1:** Received i.p. %1 Tween 80, **C2:** Received i.p. %1 Tween 80 and intragastrically tap water, **R:** Received Reserpine, **R+C:** Received Reserpine and Citalopram, **R+T:** Received Reserpine and TQ, **R+C+T:** Received Reserpine and Citalopram and TQ

#### Novelty suppressed feeding test (NSFT)

Compared to the C1 group, the amount of time to take the first bite which was recorded as the latency to feed increased in the R group (p < 0.01) and the amount of food consumed decreased in the R group (p < 0.05).

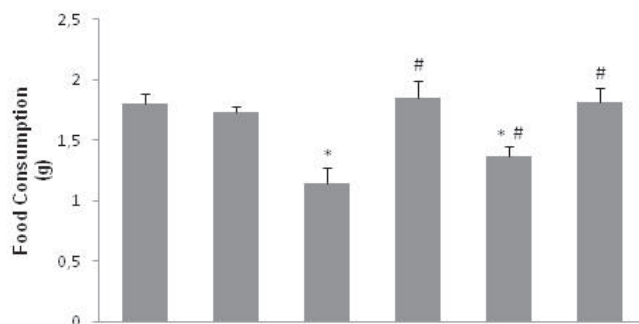
TQ, citalopram and citalopram+TQ treatment induced a reduction in the amount of time to take the first bite and an increase in the amount of food consumed (p < 0.05). However, citalopram and citalopram+TQ treatment provided a more significant recovery (p < 0.01) (The latency to feed: F (5, 30) = 444.842; the amount of food consumed: F (5, 30) = 48.248). There was no statistical difference in R+C treatment group and R+C+T treatment group (p > 0.05). (Figure 4 A, B)



**FIGURE 4A - Latency to Feed (sec)** The values are means±SD; n = 6.

\* < 0.05 versus C1 and C2 groups, \*\*p < 0.01 versus C1 groups, # < 0.05 versus R group, ## < 0.01 versus R group

**C1:** Received i.p. %1 Tween 80, **C2:** Received i.p. %1 Tween 80 and intragastrically tap water, **R:** Received Reserpine, **R+C:** Received Reserpine and Citalopram, **R+T:** Received Reserpine and TQ, **R+C+T:** Received Reserpine and Citalopram and TQ

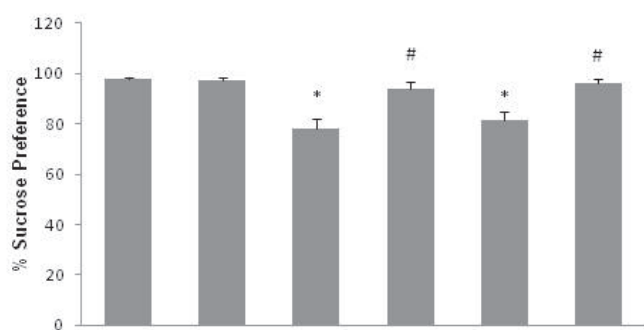


**FIGURE 4B - Food Consumption (g)** The values are means±SD; n = 6.

\* < 0.05 versus C1 and C2 groups, # < 0.05 versus R group

**C1:** Received i.p. %1 Tween 80, **C2:** Received i.p. %1 Tween 80 and intragastrically tap water, **R:** Received Reserpine, **R+C:** Received Reserpine and Citalopram, **R+T:** Received Reserpine and TQ, **R+C+T:** Received Reserpine and Citalopram and TQ





**FIGURE 4C** - % sucrose preference test. The values are means $\pm$ SD; n = 6.

\* < 0.05 versus C1 and C2 groups, # < 0.05 versus R group

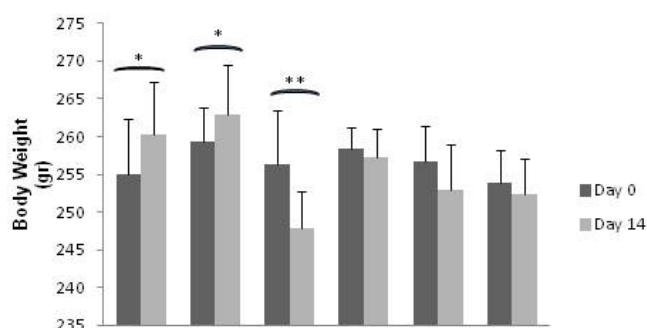
**C1:** Received i.p. %1 Tween 80, **C2:** Received i.p. %1 Tween 80 and intragastrically tap water, **R:** Received Reserpine, **R+C:** Received Reserpine and Citalopram, **R+T:** Received Reserpine and TQ, **R+C+T:** Received Reserpine and Citalopram and TQ

#### Sucrose preference test (SPT)

The sucrose preference was lower in the R group than in the C1 group ( $p < 0.05$ ), and the sucrose preference was higher in the TQ, citalopram and citalopram+TQ treatment groups than in the R group ( $p < 0.05$ ) ( $F(5, 30) = 68.689$ ). (Figure 4 C)

#### Body weight

At the end of the 14<sup>th</sup> day, while there was an increase in body weight in the C1 and C2 groups ( $p < 0.05$ ), there was a significant decrease in body weight in the R group ( $p < 0.01$ ). There were no significant changes in body weight in TQ, citalopram and citalopram+TQ treatment groups ( $p > 0.05$ ). (Figure 5)



**FIGURE 5** - Measured body weight on the 0<sup>th</sup> day and the 14<sup>th</sup> day. The values are means $\pm$ SD; n = 6.

\*  $p < 0.05$ ; \*\*  $p < 0.01$

**C1:** Received i.p. %1 Tween 80, **C2:** Received i.p. %1 Tween 80 and intragastrically tap water, **R:** Received Reserpine, **R+C:** Received Reserpine and Citalopram, **R+T:** Received Reserpine and TQ, **R+C+T:** Received Reserpine and Citalopram and TQ

#### Biochemical tests

##### Immunohistochemistry for DA, NA and 5-HT expressions

In the study, levels of DA, NA and 5-HT expressed in nuc. accumbens and hypothalamus regions in the studied rat brains were examined and the following results were obtained. DA and NA caused punctual stains on neuron cytoplasm, axonal and dendritic extensions generally in nuc. accumbens and hypothalamus. Besides, these were characterized with dark red and diffuse homogenous appearance especially in neuron cytoplasm where they were very intensively expressed. On the other hand, diffuse homogenous staining characteristic and multifocal distribution pattern of 5-HT expressions drew attention. Intensive immunopositive staining was observed in such a way that the borders of nuc. accumbens and hypothalamus neurons were apparent especially in the brains of the rats belonging to TQ, citalopram and citalopram+TQ treatment groups.

In the study, when compared with the C1 group, DA, NA and 5-HT activity in nuc. accumbens and hypothalamus regions were observed to be at the lowest level in the R group when compared to all the other groups. In C1 and C2 groups where depression was not induced, DA and NA immunoreactivities were observed at different levels mainly intense in nuc. accumbens and

5-HT activity was mainly in selective cell groups and at a lower level.

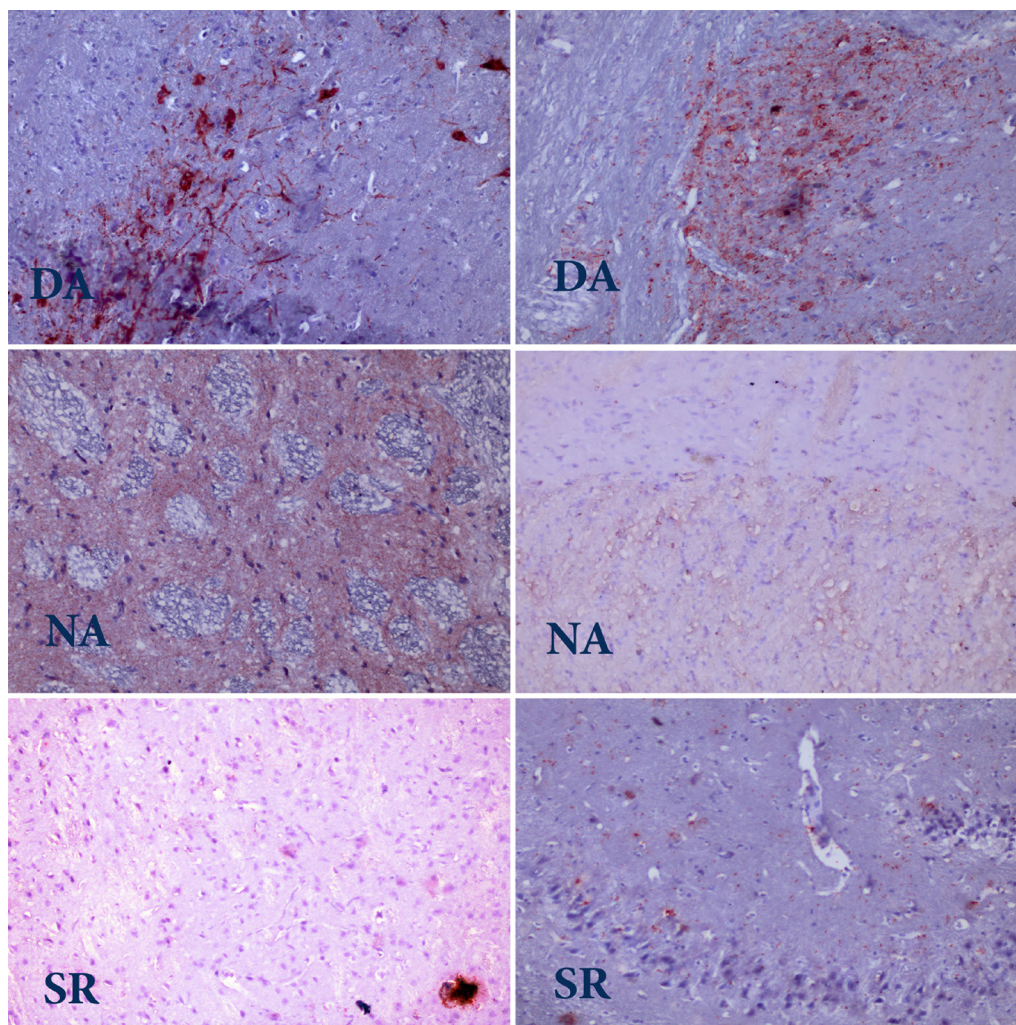
In the citalopram treatment group, significantly increasing expressions of all the three mediators were observed when compared to the R group. Specifically 5-HT activity was observed to be stronger than in the C1, C2 and R groups.

Similarly, in the TQ treatment group, DA, NA and 5-HT expressions were at a significantly higher level when compared to the R group. It was observed that they exhibited staining characteristics similar to those of the C2 group.

Again, in the TQ+citalopram treatment group, especially 5-HT activity is significantly at the highest level among all the groups and there is a significant increase when compared to the R group. Furthermore,

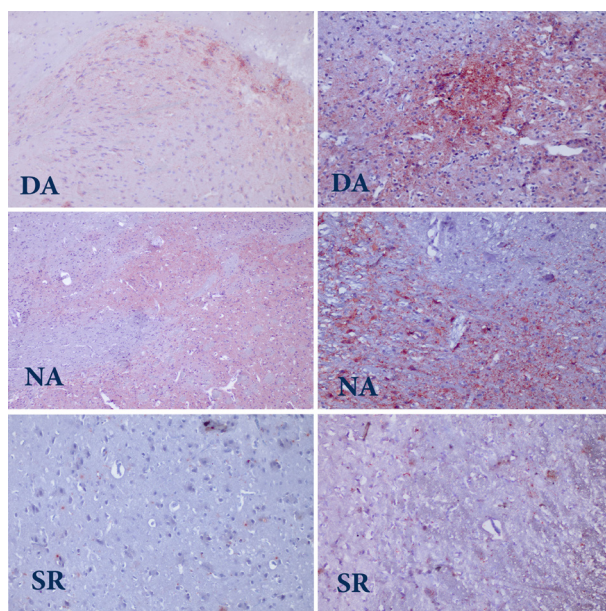
in the TQ+citalopram treatment group, DA and NA stains in nuc. accumbens and hypothalamus regions are at a higher level than those in the R group.

To sum up, in this study, it can be seen that a decrease in the level of all the mediators, which were successfully analysed with the administration of reserpine, was ensured, and that a depression-like experimental model in rats was formed. Moreover, it was observed that following the induction of depression using reserpine in the chronic period, TQ administration ensured recovery at a level similar to the control group rats in returning the depression-related changes to normal. Besides, the highest level of recovery was seen in the groups that were given TQ+citalopram, citalopram and TQ, respectively. (Figure 6a, 6b and 6c)

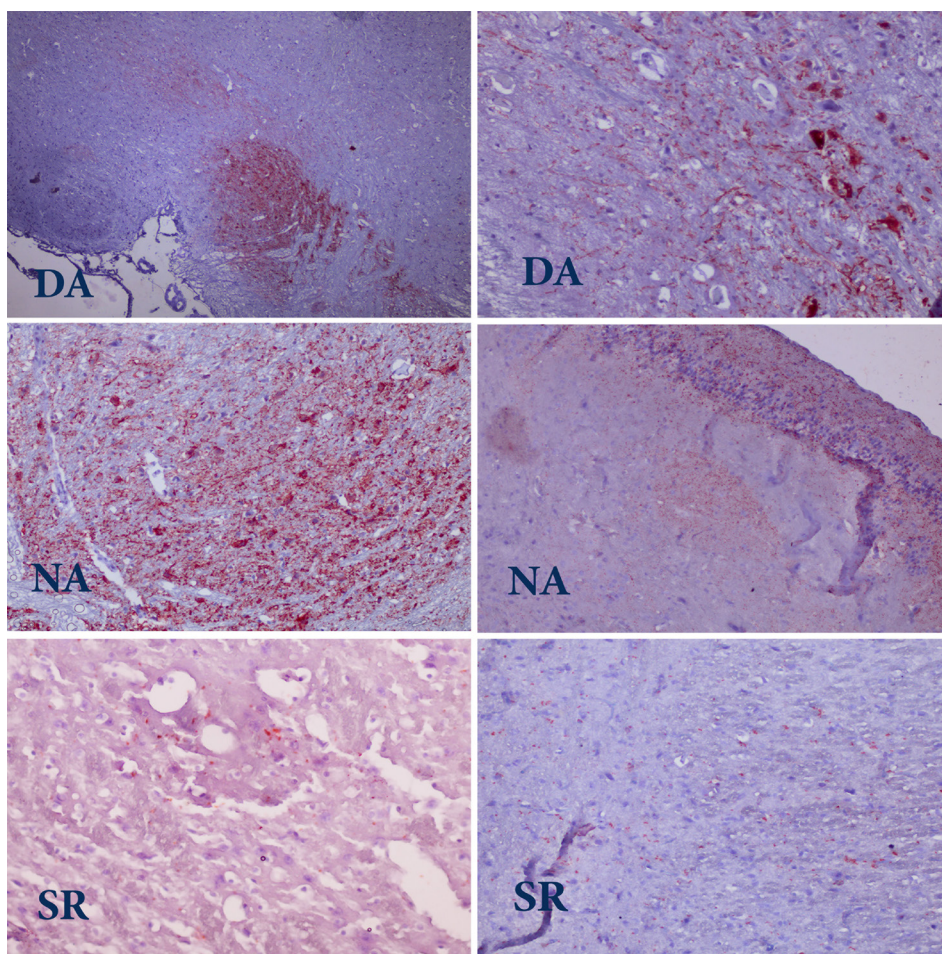


**FIGURE 6a** - Group C1 and Group C2;





**FIGURE 6b** - Group R and Group R+T;

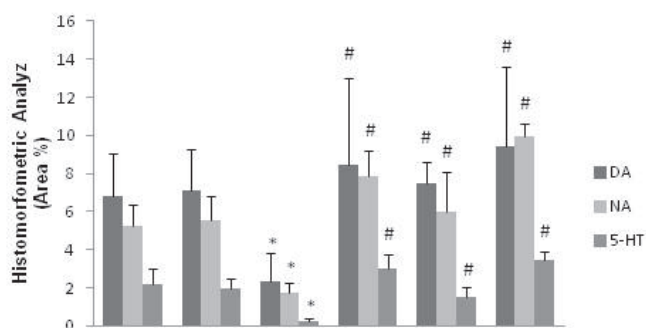


**FIGURE 6c** - Group R+C and Group R+C+T

The levels of DA, NA and SR (5-HT) expressed in nuc. accumbens and hypothalamus regions. Avidin-Biotinimmunoperoxidase test, Mayers Hematoxylen background staining.

### Quantitative Histomorphometric Analyses of DA, NA and 5-HT expressions

In the R group, the amounts of DA, NA and 5-HT decreased significantly when compared to the C1 group ( $p < 0.01$ ). In the TQ treatment group, there was a significant increase in DA and NA amounts when compared to the R group ( $p < 0.01$ ). As for the 5-HT amount, an increase was observed in the TQ treatment group when compared to the R group ( $p < 0.05$ ). DA, NA and 5-HT were found to be at higher levels in the citalopram treatment group in comparison to the R group ( $p < 0.01$ ). It was found out that DA, NA and 5-HT amounts were at a high level in the TQ+citalopram treatment group in comparison to the R group ( $p < 0.01$ ). The amounts of DA, NA and 5-HT in this group were found to be the highest of all the groups. (DA:  $F(5, 30) = 4.213$ ; NA:  $F(5, 30) = 28.738$ ; 5-HT:  $F(5, 30) = 11.589$ ). (Figure 7)



**FIGURE 7** - Histomorphometric Analysis (Area %) The values are means $\pm$ SD; n = 6.

\* $< 0.05$  versus C1 groups, # $< 0.05$  versus R group

**C1:** Received i.p. %1 Tween 80, **C2:** Received i.p. %1 Tween 80 and intragastrically tap water, **R:** Received Reserpine, **R+C:** Received Reserpine and Citalopram, **R+T:** Received Reserpine and TQ, **R+C+T:** Received Reserpine and Citalopram and TQ. The levels of MDA, NOx, TAS, TOS and GSH and RSH levels in the brain tissues and plasma

Brain cortex and plasma TOS, MDA and NOx levels of depressed rats were found to be higher than the C1 group ( $p < 0.05$ ). When the R group was compared to the C1 group, it was observed that brain cortex GSH, plasma RSH levels and TAS in brain cortex and plasma decreased ( $p < 0.05$ ). In the groups that were treated only with TQ and only with citalopram, brain cortex and plasma TOS, MDA and NOx levels decreased while TAS increased ( $p < 0.05$ ). There was an increase in brain cortex GSH and plasma RSH levels ( $p < 0.05$ ). The combined administration of TQ+citalopram to rats caused a significant increase in the activity of brain cortex GSH and plasma RSH compared with the groups of animals receiving only citalopram or TQ ( $p < 0.05$ ). The simultaneous application of TQ+citalopram decreased the MDA, NOx levels and TOS in brain cortex and plasma when compared with rats receiving only TQ or citalopram ( $p < 0.05$ ). (Brain cortex NOx:  $F(5, 30) = 142.356$ ; MDA:  $F(5, 30) = 235.623$ ; GSH:  $F(5, 30) = 105.573$ ; TAS:  $F(5, 30) = 94.220$ ; TOS:  $F(5, 30) = 70.604$ ) (Plasma NOx:  $F(5, 30) = 95.341$ ; MDA:  $F(5, 30) = 243.970$ ; RSH:  $F(5, 30) = 97.032$ ; TOS:  $F(5, 30) = 30.866$ ; TAS:  $F(5, 30) = 58.291$ ). Brain cortex and plasma biomarker levels were presented in Table II and Table III, respectively.

**TABLE II** - The results of brain cortex MDA, NOx, GSH levels, TAS and TOS

Brain Cortex					
	MDA Levels (nmol/g)	NOx Levels ( $\mu$ mol/g)	GSH Levels (nmol/g)	TAS (pg/mg)	TOS (U/mg)
<b>C1</b>	4,35 $\pm$ 0,25	0,34 $\pm$ 0,01	4,05 $\pm$ 0,10	24,91 $\pm$ 0,87	3,07 $\pm$ 0,15
<b>C2</b>	4,6 $\pm$ 0,20	0,35 $\pm$ 0,01	3,96 $\pm$ 0,05	24,73 $\pm$ 0,69	3,20 $\pm$ 0,14
<b>R</b>	7,01 $\pm$ 0,21 *	0,54 $\pm$ 0,03 *	2,08 $\pm$ 0,07 *	20,62 $\pm$ 0,49 *	6,29 $\pm$ 0,20 *
<b>R+C</b>	4,83 $\pm$ 0,08 #	0,40 $\pm$ 0,01 #	3,87 $\pm$ 0,14 #	26,17 $\pm$ 0,77 #	4,38 $\pm$ 0,28 #
<b>R+T</b>	4,38 $\pm$ 0,23 #	0,37 $\pm$ 0,01 #	4,02 $\pm$ 0,13 #	27,92 $\pm$ 0,55 #	4,04 $\pm$ 0,17 #
<b>R+C+T</b>	3,97 $\pm$ 0,10 #	0,36 $\pm$ 0,01 #	4,18 $\pm$ 0,11 #	29,59 $\pm$ 0,55 #	3,91 $\pm$ 0,04 #

The values are means $\pm$ SD; n = 6.

\* p< 0.05 Significant differences with C1 group; # p<0.05 Significant differences with R group

**C1:** Received i.p. %1 Tween 80, **C2:** Received i.p. %1 Tween 80 and intragastrically tap water, **R:** Received Reserpine, **R+C:** Received Reserpine and Citalopram, **R+T:** Received Reserpine and TQ, **R+C+T:** Received Reserpine and Citalopram and TQ

**TABLE III** - The results of plasma MDA, NOx, GSH levels, TAS and TOS

Plasma					
	MDA Levels (nmol/ml)	NOx Levels ( $\mu$ mol/ml)	RSH Levels (nmol/ml)	TAS (pg/ml)	TOS (U/ml)
<b>C1</b>	0,48 $\pm$ 0,015	8 $\pm$ 0,14	154,81 $\pm$ 1,13	31,94 $\pm$ 1,32	3,21 $\pm$ 0,13
<b>C2</b>	0,51 $\pm$ 0,009	8,46 $\pm$ 0,35	152,91 $\pm$ 5,19	31,5 $\pm$ 1,04	3,50 $\pm$ 0,21
<b>R</b>	0,72 $\pm$ 0,013 *	12,01 $\pm$ 0,47 *	131,9 $\pm$ 0,78 *	25,05 $\pm$ 0,55 *	5,83 $\pm$ 0,15 *
<b>R+C</b>	0,50 $\pm$ 0,007 #	8,83 $\pm$ 0,28 #	152,5 $\pm$ 2,25 #	27,16 $\pm$ 0,81 #	4,24 $\pm$ 0,15 #
<b>R+T</b>	0,49 $\pm$ 0,007 #	9,25 $\pm$ 0,41 #	155,83 $\pm$ 1,47 #	27,58 $\pm$ 0,74 #	4,05 $\pm$ 0,20 #
<b>R+C+T</b>	0,47 $\pm$ 0,015 #	8,54 $\pm$ 0,32 #	156,5 $\pm$ 1,37 #	28,11 $\pm$ 0,76 #	3,87 $\pm$ 0,22 #

The values are means $\pm$ SD; n = 6.

\* p< 0.05 Significant differences with C1 group; # p<0.05 Significant differences with R group

**C1:** Received i.p. %1 Tween 80, **C2:** Received i.p. %1 Tween 80 and intragastrically tap water, **R:** Received Reserpine, **R+C:** Received Reserpine and Citalopram, **R+T:** Received Reserpine and TQ, **R+C+T:** Received Reserpine and Citalopram and TQ

## DISCUSSION

In this study, our objective is to examine the antidepressant and antioxidant activity of TQ in a reserpine-induced depression model and to investigate the antidepressant and antioxidant activity of the combined treatment of TQ+citalopram.

Based on the results, i.p. reserpine injection of 0.2 mg/kg for 14 days significantly increased the immobility time and decreased the climbing and swimming time in the forced swimming test. In the tail suspension test, reserpine increased total duration of immobility and average duration of immobility episode. Besides, in the novelty suppressed feeding test, the latency to



feed increased, whereas food consumption decreased. % sucrose preference decreased. At the end of 14 days, body weights of the rats decreased significantly. Other studies have also used reserpine to induce depression-like disorders. In one study, 5 mg/kg i.p. reserpine was used to induce depression-like disorders and increased immobility time during the FST was reported (Bakhtiarpoor, Mahbubeh, Kaffashian, 2018). A significant decrease in the food intake and the body weight of the rats that were administered i.p. reserpine injection of 0.5 mg/kg for 10 days was reported. An increase in immobility time in the FST and TST was found (Park *et al.*, 2018). Reserpine is the blocker of vacuolar monoamine reuptake that can result in the evacuation of monoamines in the brain, which subsequently can lead to depressive-like symptoms in animals. An increase in the latency to feed and a decrease in food consumption in the NSFT were reported in rats that were injected with 1 mg/kg subcutaneous (sc) reserpine for 3 days (Blasco-Serra *et al.* 2015). It was found that i.p. 2 mg/kg and 4 mg/kg reserpine injection in rats decreased SPT significantly (Skalisz *et al.*, 2002; Ozerov *et al.*, 2016).

14 days of treatment with TQ at doses of 10 mg/kg significantly ameliorated the reserpine-induced changes. TQ decreased the immobility time and increased the climbing and swimming time in the FST. In the TST, TQ decreased total duration of immobility and average duration of immobility episode. In the NSFT, latency to feed decreased while food consumption increased. % sucrose preference increased. At the end of 14 days, there were no significant changes in body weight. Studies in the literature have yielded results similar to ours. However, while those studies are on the acute effects (single dose) of TQ on behavioral tests, our study is on the long term effects of TQ on reserpine-induced depression for 14 days.

Regarding depression-like behaviors induced by lipopolysaccharide, a single dose ip. 40 mg/kg TQ injection brought immobility time in the FST closer to the control group. Therefore, it was reported that TQ could have protective effects against depression and beneficial effects on the nervous system (Hosseini *et al.* 2012). It was also reported that the injection of ip. 20 mg/kg TQ

to the acute stress model formed in rats with FST and TST increased the swimming and climbing time and decreased the immobility time when compared to the group to which TQ was not administered (Aquib, Najmi, Akhtar, 2015). 10 and 20 mg/kg TQ administered with 20 mg/kg fluoxetine for 21 days decreased immobility time and increased locomotor activity in diabetic rats. It has been reported that the use of TQ in combination with fluoxetine may be useful in controlling diabetes-induced depression (Safhi *et al.*, 2019).

In our study, citalopram and TQ+citalopram treatment demonstrated better recovery in comparison to TQ treatment in behavioral tests. No differences were observed with regard to the effects of citalopram and TQ+citalopram treatment on behavioral tests. In the literature, there are no studies regarding the effects of combined treatment of TQ+citalopram on behavioral tests in the stress or depression models formed. The results of the studies performed regarding the effects of citalopram on the experimental stress model formed support our findings. A group of rats was exposed to chronic unpredictable stress experimentally for 4 weeks and in the following 3 weeks, 5, 10 and 20 mg/kg citalopram treatment was applied. It was reported that 10 and 20 mg/kg citalopram treatment brought % sucrose preference, immobility time, swimming time and climbing time to the levels of the control group but did not cause any changes in body weight (Yang *et al.*, 2013). In an acute stress study performed with FST in rats, a single dose of 20 mg/kg citalopram i.p. injection prior to FST was shown to result in an increase in swimming time and a decrease in immobility time. It is argued that citalopram treatment is effective in improving social stress related to depressive disorder (Ara, Bano, 2012).

One of the mechanisms that cause depression is the oxidative stress increasing in the brain. It has been reported that reserpine can cause depression by increasing oxidative damage (Bakhtiarpoor, Mahbubeh, Kaffashian, 2018). The results of this study indicate that reserpine decreased brain cortex and plasma TAS levels significantly. GSH levels in the brain cortex and RSH levels in plasma decreased. Reserpine increased TOS, MDA and NOx levels in the brain cortex and plasma. Although studies in the literature include 3-day sc.

injection of reserpine at a dose of 1 mg/kg, our results are consistent with the results of other researchers who reported an increase in the marker of oxidative stress of reserpine (Arora, Chopra, 2013; Nade *et al.*, 2013; Wang *et al.*, 2015). Reserpine increased MDA and NOx levels in brain tissue and decreased GSH levels. Therefore, reserpine is reported to cause depression by inducing oxidative-nitrosative stress in rat brain. 5 mg/kg i.p. reserpine to induce depression-like disorders was found to have caused an increase in plasma and brain tissue MDA levels and a decrease in TAS (Bakhtiarpoor, Mahbubeh, Kaffashian, 2018).

14-day use of TQ at 10 mg/kg increased the reserpine-induced reduction in the brain cortex and plasma TAS. Brain cortex GSH and plasma RSH levels increased. TQ decreased the reserpine-induced increase in brain and plasma TOS, MDA and NOx levels. I.p. 20 mg/kg TQ injected half an hour before exposure to stress in an acute stress model in rats formed with FST and TST resulted in a decrease in TBARS levels and brought glutathione level to normal levels (Aquib, Najmi, Akhtar, 2015). In a study conducted by Gilhotra and Dhingra (2011), following 6-hour immobilization stress in rats, it was observed that the increased NOx levels in the brain tissue of the stressed group decreased with the administration of 20 mg/kg (ip) TQ. In another study, it was observed that in a case of neurotoxicity with 20 mg/kg arsenic taken through drinking water for 21 days in rats, MDA and NOx levels in cerebral cortex, cerebellum and brain stem increased and GSH levels decreased. A decrease in MDA and NOx levels and an increase in GSH levels were observed in rats that were treated with 10 mg/kg TQ 1 hour after arsenic exposure. According to the results of the study, TQ was reported to be a strong antioxidant and an agent protecting from toxicity (Kassab, El-Hennamy, 2017). In a study performed by Safhi *et al.*, 2019, 10 and 20 mg/kg TQ administered with 20 mg/kg fluoxetine for 21 days decreased blood glucose, TBARS and interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis alpha (TNF- $\alpha$ ) levels and increased GSH and antioxidant enzyme levels in diabetic rats. Therefore, it was reported that TQ in combination with fluoxetine may be useful in controlling depression aggravated by diabetes.

In this study, when compared to treatments with TQ as a single agent and citalopram as a single agent, citalopram + TQ treatment caused a further decrease in TOS, MDA and NOx levels in cerebral cortex and plasma and a further increase in TAS, GSH and RSH levels. A better recovery was obtained against oxidative stress caused by reserpine. However, there are no studies on the effects of citalopram+TQ on oxidative stress and antioxidant parameters in literature. In this study, treatment with 10 mg/kg citalopram as a single agent for 14 days provided protection from oxidative stress caused by reserpine. This treatment resulted in a decrease in TOS, MDA and NOx levels in cerebral cortex and plasma and an increase in TAS. There was also an increase in brain cortex GSH and plasma RSH levels. In literature, it was stated that in patients with major depression, treatment with 20 mg/day citalopram for 12 weeks brought the increased MDA levels closer to those of the control group (Khanzode *et al.*, 2003). However, a study by Herbet *et al.* (2016), reported that the i.p. injection of 10 mg/kg citalopram for 14 days did not cause a change in the plasma TAS levels of healthy rats.

Monoamine neurotransmitters in the central nervous system play a vital role in depression. Reserpine blocks amine storage processes. In this study, i.p. injection of 0.2 mg/kg reserpine for 14 days caused a decrease in NA, DA and 5-HT levels in nuc. accumbens and hypothalamus. A group of rats were given 0.5 mg/kg i.p. reserpine injection for 10 days and at the end of the 10<sup>th</sup> day, a decrease in plasma 5-HT levels was reported (Park *et al.*, 2018). It was reported that cerebral cortex NA, DA and 5-HT levels decreased significantly in rats to which 1 mg/kg sc reserpine injection was administered for 3 consecutive days (Arora, Chopra, 2013). For this reason, reserpine has been reported to cause depression by inducing neurochemical changes. 1 mg/kg sc. reserpine injection for 3 days caused a decrease in basolateral amigdala (BLA) NA, DA and 5-HT levels in rats. It was found that in reserpinized rats, the increase in oxidative stress and the decrease in biogenic amines induced depression (Liu *et al.*, 2014). A significant decrease in NA, DA and 5-HT levels in nuc. accumbens and hypothalamus was observed in rats to which 0,2 mg/kg reserpine was administered (Antkiewicz-Michaluk *et al.*, 2014).

Treatment with 10 mg/kg TQ for 14 days increased NA, DA and 5-HT levels in nuc. accumbens and hypothalamus. Studies in literature support our study. I.p. injection of 20 mg/kg TQ to rats in an acute stress model formed with FST and TST half an hour before exposure to stress caused an increase in 5-HT levels in the brain (Aquib, Najmi, Akhtar, 2015). 20 mg/kg TQ administered to rats by Alam *et al.* (2020), decreased Indoleamine-2,3-dioxygenase activation, causing an increase in hippocampal 5-HT levels and a decrease in IL-6 ve TNF- $\alpha$  levels and it was suggested that it can have antidepressant effects.

Based on the results, 10 mg/kg citalopram+TQ treatment for 14 days yielded a further increase in NA, DA and 5-HT levels in nuc. accumbens and hypothalamus when compared to the groups to which only 10 mg/kg citalopram and only 10 mg/kg TQ treatment was given. In literature, there are no studies on the effects of citalopram + TQ treatment on NA, DA and 5-HT levels. Treatment with 10 mg/kg citalopram for 14 days resulted in an increase in NA, DA and 5-HT levels in nuc. accumbens and hypothalamus. Our study demonstrates the 14-day (long term) effect of citalopram. Intragastric treatment with 1,8 mg/kg citalopram for 30 days resulted in an increase in DA, NA and 5-HT levels (Feng *et al.*, 2018). Studies on the effects of short term citalopram treatment in the literature also support the findings of our study. In rats stressed with FST following a single dose of 20 mg/kg citalopram ip. injection, an increase in hypothalamus, hippocampus and amigdala 5-HT levels was observed in rats that were injected with citalopram when compared to those that were not (Ara, Bano, 2012). Subcutaneous injection of 5 mg/kg citalopram as 2 doses resulted in an increase in NA and 5-HT levels in medial prefrontal cortex, and in DA levels in nuc. accumbens (Bjorkholm *et al.*, 2015).

In this study, both TQ and TQ+citalopram resulted in an improvement in some parameters in reserpine-induced depression. TQ+citalopram treatment further increased antioxidant capacity and monoamine amount. Some of the observed antidepressant effects of TQ can be attributed to its anti-oxidant activity. However, it is recommended that the role of monoaminergic systems in the antidepressant effect of TQ be further studied using different agonists and antagonists.

## CONCLUSION

It is suggested that TQ has the ability to prevent reserpine-induced depression. 14-day TQ treatment increased antioxidant capacity and monoamine amount and ensured improvement in behavioral tests. Its mechanism responsible for antidepressant activity may be attributed to its increasing antioxidant capacity.

However, further studies are needed in order to explain the mechanism causing an increase in the amount of monoamines. The improving effects of citalopram+TQ combined treatment on behavioral tests and its effects on antioxidant capacity and monoamine amount were more when compared to treatment with TQ as a single agent. For this reason, in order to prevent reserpine-induced depression, both TQ and citalopram+TQ combination can be used.

## AUTHOR CONTRIBUTION STATEMENT

DK, ED and ÇÖ conceived and designed the research. DK conducted experiments. DK analyzed data. DK wrote the manuscript. All authors read and approved the manuscript.

## ETHICAL STATEMENT

All procedures in this study were performed in accordance with the Gazi University Laboratory Animals and Experimental Researches Center. Animals were approved by the Gazi University Ethical and Research Committee (Approval No: G.Ü.ET-16.012).

## ACKNOWLEDGMENTS

This work was supported by the Ahi Evran University Scientific Research Projects Coordination Unit. Project Number: TIP.A3.17.001.

## CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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Received for publication on 13<sup>rd</sup> November 2019

Accepted for publication on 16<sup>th</sup> June 2020