

# Altered ratio of proapoptotic and antiapoptotic proteins in different brain regions of female rats in model of post-traumatic stress disorder

[Post-travmatik stres hastalığı modelinde dişi sıçanların farklı beyin bölgelerinde proapoptotik ve antiapoptotik proteinlerin oranındaki değişim]

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## ABSTRACT

**Objective:** The B-cell lymphoma/leukemia-2 (Bcl-2) family of proteins governs mitochondrial membrane permeability where the programmed apoptotic process is controlled by the balance between proapoptotic (Bax) and antiapoptotic (Bcl-2) proteins. We aimed to investigate the [Bcl-2]/[Bax] in different brain regions in a post-traumatic stress disorder rat model.

**Methods:** Female Sprague-Dawley rats were exposed to dirty cat litter (trauma) for 10 min and the protocol was repeated 1 week later with a trauma reminder (clean litter) in reversed 12 h light/dark cycle. The rats received intraperitoneal saline, fluoxetine (2.5 mg/kg/day) or propranolol (10 mg/kg/day) for 7 days between exposure sessions. Following exposure to the trauma reminder, elevated plus maze experiments were done. Immunoblotting was used to quantify [Bcl-2] and [Bax] proteins in the homogenates of the dorsal hippocampus, the frontal cortex and the amygdaloid complex.

**Results:** Fluoxetine reversed the increases in the anxiety indices and the freezing times. In the amygdaloid complex and the frontal cortex, the [Bcl-2]/[Bax] decreased in the traumatized control rats significantly ( $p < 0.0001$ ), but not in the dorsal hippocampus. Although the fluoxetine treatment reversed the apoptotic changes but propranolol failed and caused proapoptotic proteins to increase.

**Conclusion:** These results may suggest a neuroprotective role for fluoxetine but not for propranolol.

**Key Words:** Dorsal hippocampus, amygdaloid complex, frontal cortex, Bcl-2, Bax, predator scent test, Western blot, cat litter

**Conflict of Interest:** The authors have no conflict of interest.

## ÖZET

**Amaç:** B-hücre lenfoma/lösemi-2 (Bcl-2) protein ailesi, proapoptotik (Bax) ve antiapoptotik (Bcl-2) proteinleri arasındaki denge ile kontrol edilen programlı hücre ölümü işleminde mitokondri zar geçirgenliğini düzenlemektedir. Post travmatik stres sıçan modelinde farklı beyin bölgelerinde [Bcl-2]/[Bax] oranını araştırmayı amaçlıyoruz.

**Metod:** Dişi Sprague-Dawley sıçanlar ters 12 s aydınlık/karanlık siklusunda 10 dak. kirli kedi kumuna maruz bırakıldı ve protokol 1 hafta sonra travma çağrıştırıcı (temiz kum) ile tekrarlandı. Sıçanlara intraperitoneal tuzlu su, fluoksetin (2.5 mg/kg/day) veya propranolol (10 mg/kg/gün) uygulama dönemleri arasında 7 gün verildi. Travma çağrıştırıcıya maruz bırakmanın ardından yükseltilmiş artı labirent yapıldı. Western blot dorsal hipokampus, frontal korteks ve amigdalar bölge homojenatlarında Bcl-2 ve Bax proteinlerinin miktarını belirlemede kullanıldı.

**Bulgular:** Fluoksetin, anksiyete indeksleri ve dona kalma sürelerindeki artışları geri çevirdi. Amigdalar bölgede ve frontal korteks de [Bcl-2]/[Bax] oranı travmatik kontrol sıçanlarda anlamlı olarak azaldı ( $p < 0.0001$ ), fakat dorsal hipokampusda azalmadı. Fluoksetin apoptotik değişimleri geri çevirmesine rağmen propranolol çevirmedi ve proapoptotik proteinde artışa sebep oldu.

**Sonuç:** Bu sonuçlarla fluoksetinin nöroprotektif rolü önerilebilir fakat propranolol için önerilemez.

**Anahtar Kelimeler:** Dorsal hipokampus, amigdalar bölge, frontal korteks, Bcl-2, Bax, saldırgan hayvan koku testi, Western blot, kedi kumu

**Çıkar Çatışması:** Yazarların çıkar çatışması yoktur.

## Introduction

Post-traumatic stress disorder (PTSD) is a stress-related mental disorder caused by an experience of a traumatic event. Affected individuals have intrusive re-experiences of the traumatic events that lack awareness of the context and the time [1]. The episodes of re-experiences aggravate and maintain the disease and the symptoms, since the affected individual re-experiences trauma as if it was happening in the present moment [1]. Neuroanatomical studies have revealed critical involvement of three important brain regions, the hippocampus, the prefrontal cortex and the amygdala. It was stated that PTSD is associated with hypoactivity in the ventromedial prefrontal cortex, hyperactivity in the amygdala and reduced volume in the hippocampus [2]. We can assume that the hippocampus fails during stress and cannot place the memories in the correct context of space and time. The flashbacks, the most characteristic symptom, occur as a consequence of this hippocampal failure. In literature, many studies on PTSD concern the volume changes of particular brain regions and try to account for the pathology of the disease [3-5]. A monozygotic twin study performed in veterans revealed that hippocampal asymmetry is associated with PTSD [6]. However, Jatzko et al. [7] demonstrated no change in the hippocampal volumes of patients with chronic PTSD, traumatized in a plane crash in 1988 (Ramstein, Germany) compared to that of controls [6,7]. Interestingly, a cross-sectional magnetic resonance study performed in the veterans of Vietnam Conflict and Persian Gulf War has shown increased amygdala volume of the affected subjects [8].

Apoptosis that occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues that it can also be proposed to contribute to the structural alterations in the stress-related mood disorders [9-11]. The apoptotic process is programmed and critically controlled by the balance between proapoptotic and antiapoptotic proteins within the cell [12]. The B-cell lymphoma/leukemia-2 (Bcl-2) family of proteins governs mitochondrial membrane permeability and can be either proapoptotic or antiapoptotic [9]. A total of 25 genes have been identified in the Bcl-2 family where some of the proteins were antiapoptotic (Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG) and some were proapoptotic (Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk; [9]. The main mechanism of action of the Bcl-2 family of proteins is the regulation of cytochrome c release from the mitochondria via alteration of mitochondrial membrane permeability. Apoptotic stimuli cause the translocation of (Bax) from the cytosol to mitochondria, leading to multimerization, integration and cytochrome c release, caspase activation and apoptosis [13-15]. Therefore, quantitative analyses of [Bcl-2] and [Bax] can be made and a decrease in the ratio of [Bcl-2] to [Bax] may indicate apoptotic changes.

Predator scent test, a useful method serving to study the

pathology of the disease [16] and our previous data indicated that the cat litter test is a reproducible experiment where the rats subjected to dirty cat litter (the trauma) had longer freezing times and higher anxiety indices when exposed to the situational reminder, the clean cat litter [17]. Our previous another data showed that noradrenaline (NA) content in the rostral pons increased in traumatized rats with cat litter and this increase may serve as an additional neurochemical evidence for the model. Pirenzepine suppressed the anxiety index but it was not that effective in decreasing the prolonged freezing times [18].

In this current study, we wanted to demonstrate the ratio between proapoptotic and antiapoptotic proteins in the dorsal hippocampus, the amygdaloid complex and the frontal cortex of female rats traumatized with cat litter. The effects of fluoxetine or propranolol on behavioral parameters and neuronal apoptosis were also investigated.

## Materials and Methods

### Materials

The Bcl-2 and Bax specific antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary antibodies were purchased from Sigma (St Louis, MO). All chemicals were obtained from Sigma, unless stated otherwise.

### Drugs and solutions

The rats received physiological saline, fluoxetine (2.5 mg/kg; Eli Lilly, Turkey) or propranolol (10 mg/kg; Sigma, USA) for 1 week. The drugs were dissolved in physiological saline. The treatments were given once daily, at the same time of the day.

### Animals and experimental condition

An approval from the institutional ethical committee was obtained before the experiments were started (MÜHDEK approval no: 11.11.2010-88.2010.mar). Female Sprague-Dawley rats weighing 200-250 g supplied from Marmara University Animal Center (DEHAMER) were used in the study. The rats were habituated to the housing conditions for 10 days with a reversed 12 h light/dark cycle at  $21\pm 3^{\circ}\text{C}$  and  $50\pm 5\%$  humidity with unlimited access to standard rat chow and water. All experiments were performed in the dark phase at 10:00 h a.m. using a dim light source.

### Predator scent test

The stress paradigm was produced by placing the rats on 125 ml of dirty cat litter for 10 min in a plexiglass cage (30 cm X 30 cm X 40 cm). The cat litter had been used for 2 days by the same cat and had been sifted for stools as described previously [17,19-21]. The control animals were exposed to fresh, unused litter for the same amount of time. Clean cat litter was used as situational reminder and the rats were subjected to the reminder 1 week following the onset of the stress. The behavioral experiments were recorded using an overhead video camera and behavioral parameters were scored from the recordings

later. Intraperitoneal treatments were given 10 min before the predator test.

### Elevated plus maze experiments

The rats were placed on an elevated plus maze for 5 min immediately after they had been subjected to the situational reminder. The elevated plus maze had two open (50 cm X 10 cm) and two closed (50 cm X 10 cm) arms. The closed arms were surrounded by 40 cm long walls. The height of the maze was 50 cm from the ground. The labyrinth was cleaned with 5% alcohol solution before the rats were placed in it. Each rat was placed in the central square of the plus maze facing the open arms. An arm entry was defined as an animal entering the arm with all four feet and the number of entries into open and enclosed arms was scored as described previously [17,22]. The anxiety index ( $N_{\text{anxiety}}$ ) was calculated by using the following parameters and the formula:

a=cumulative time spent in open arms (sec)

b=open arm entries

c=total arm entries

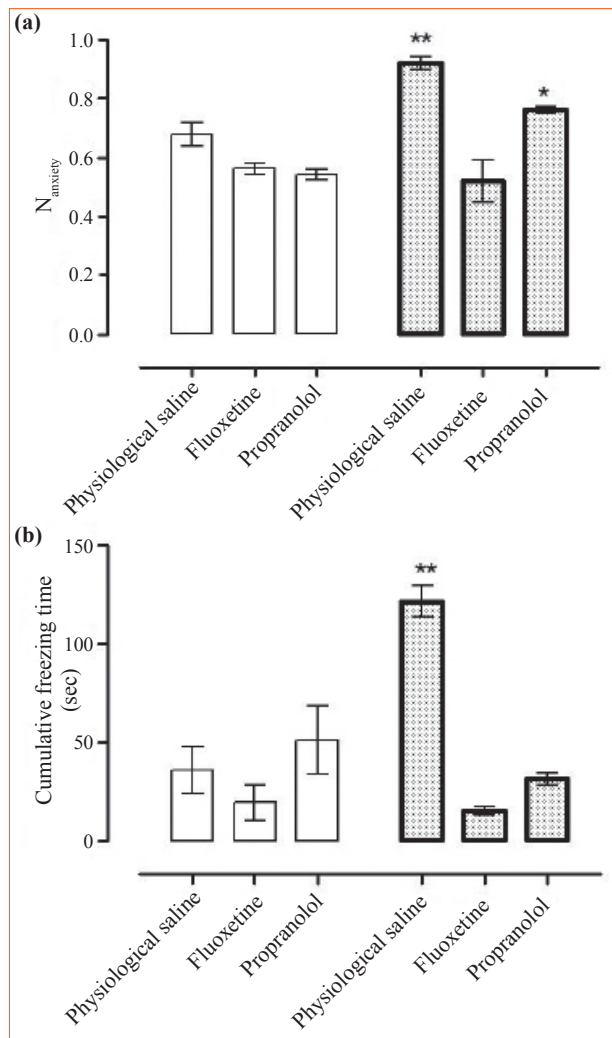
$$N_{\text{anxiety}} = 1 - \frac{1}{2} [(a / 300 \text{ sec}) + (b / c)]$$

The cumulative freezing time, a fear parameter, was also recorded and evaluated. Upon completion of the experiments the most affected rats were sacrificed with a high dose of pentobarbital and the selected brain regions were dissected and kept at  $-80^{\circ}\text{C}$  for immunoblotting.

### Tissue preparation and immunoblotting

After rapid decapitation, the hippocampus, the frontal cortex and the amygdaloid complex were removed in accordance with the Rat Brain Atlas [23] and were frozen at  $-80^{\circ}\text{C}$  until further preparation. Anteroposterior planes used for removing the frontal cortex, the dorsal hippocampus and the amygdaloid complex were located between 13.20-11.20 mm, 6.70-4.70 mm and 7.20-5.70 mm anterior to the interaural line, respectively. The interaural line was accepted as 9.00 mm posterior to the tip of the brain as indicated [23]. The frozen tissues were weighed and homogenized in ice-cold 10 mM Tris-HCl (pH=7.2) buffer containing 1 mM EDTA and protease inhibitors (0.2 mM PMSF, 1  $\mu\text{g}/\text{ml}$  leupeptin, 1  $\mu\text{M}$  pepstatin, 10  $\mu\text{g}/\text{ml}$  soybean trypsin inhibitors) with Ultraturrax homogenizer. Whole homogenates were used in Western blots. The protein content of the whole homogenate was determined as indicated [24]. Fifty  $\mu\text{g}$  of protein was loaded onto 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and electrophoretically transferred onto nitrocellulose membranes (Schleicher and Schuell, 0.45  $\mu\text{m}$ , Germany) for 120 min at 80 V. The membranes were blocked with tris buffered saline containing 1% bovine serum albumin and 0.05% Tween-20 at room temperature for 60 min and incubated overnight at  $4^{\circ}\text{C}$  with antibodies against (Bcl-2) and (Bax) proteins.

The Bcl-2 and Bax specific antibodies were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).



**Figure 1.** The effect of physiological saline, fluoxetine (2.5 mg/kg) or propranolol (10 mg/kg) on the behavioral parameters measured on elevated plus maze (n=9 per group): the anxiety indices ( $N_{\text{anxiety}}$ ) and the cumulative freezing time (**b**). The bars with thick borders represent the rats subjected to the predator scent (dirty cat litter) and the trauma reminder (clean litter) one week later. The control rats represented by bars having thin borders were only exposed to clean litter in two sessions. The treatments continued for one week between the 2 exposure sessions. \* $p < 0.05$ ; \*\* $p < 0.001$  (Bonferroni post hoc test results indicating the difference from the non-traumatized rats).

The secondary antibodies were purchased from Sigma (St Louis, MO, USA) and Pierce Biotechnology, Inc. (Rockford, IL, USA). All chemicals were obtained from Sigma, unless stated otherwise.

The blots were washed three times with TBS containing 0.05% Tween-20 (TBS-T) and incubated with alkaline phosphatase-conjugated secondary antibodies for 1 h at room temperature ( $20^{\circ}\text{C}$ ). The antibody-antigen complex was detected with NBT-BCIP. The apparent molecular weights of Bcl-2 and Bax are 26 kDa, 23 kDa, respectively. The densitometric analyses were carried out with Bio-Rad Molecular Analyst software (free edition, www.totallab.com).

## Statistical analysis

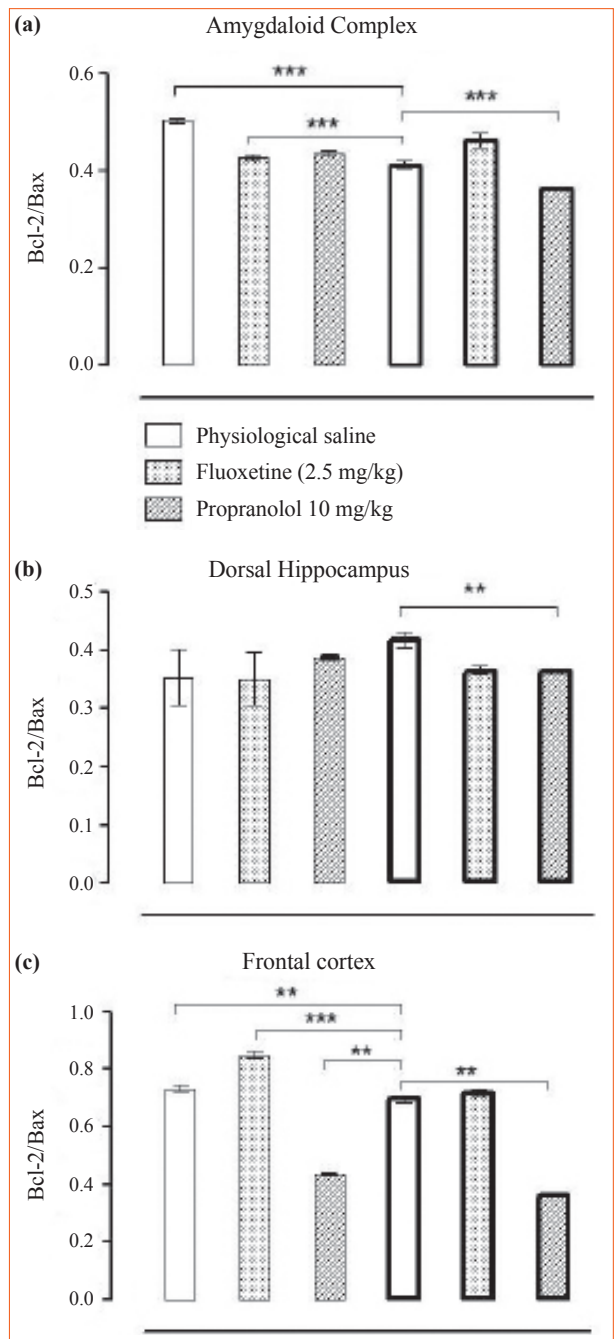
All data are expressed as mean±SEM. Two-way analysis of variance and the Bonferroni *post hoc* test were used for the analysis of data. For all statistical calculations, significance was considered to be a value of  $p < 0.05$ .

## Results

The effects of agents on behavioral parameters. The integrative index,  $N_{\text{anxiety}}$  was calculated by using the behavioral parameters collected from the elevated plus maze experiments following the second exposure to the dirty cat litter ( $n=9$  per group). Two-way analysis of variance detected that there is significant variance between the groups and a significant interaction between trauma and the treatments ( $df=2$ ,  $F=7.524$ ;  $p=0.0016$ ). The source of variation was found to be significant for both the trauma ( $df=1$ ,  $F=14.54$ ;  $p=0.0005$ ) and the treatments ( $df=2$ ,  $F=21.87$ ;  $p<0.0001$ ). Bonferroni *post hoc* test revealed that the  $N_{\text{anxiety}}$  calculated in the traumatized control rats that received saline treatment was significantly higher than the non-traumatized group ( $p<0.001$ ). The  $N_{\text{anxiety}}$  of traumatized rats treated with fluoxetine was not found to be different from the non-traumatized control rats (Fig. 1a). However, propranolol treatment increased the  $N_{\text{anxiety}}$  ( $p<0.05$ ). When another index of anxiety, the cumulative freezing time was evaluated by two-way analysis of variance, both trauma ( $df=1$ ,  $F=4.716$ ;  $p=0.0463$ ) and the treatments ( $df=2$ ,  $F=14.22$ ;  $p=0.0003$ ) were found to produce the variation and there was a significant interaction between the trauma and the treatments ( $df=2$ ,  $F=12.02$ ;  $p=0.0008$ ). Bonferroni *post hoc* test revealed that the cumulative freezing time in the traumatized control rats that received saline treatment was significantly higher than the non-traumatized group ( $p<0.001$ ). Both drugs decreased the freezing times in traumatized rats (Fig. 1b).

**Immunoblotting Analyses.** We performed immunoblotting experiments in 4 rats whose anxiety index was calculated as 1 individually. The homogenates of the dorsal hippocampus, the frontal cortex and the amygdaloid complex were run in polyacrilamide gel and Western blots were collected for Bcl-2 and Bax proteins as shown in Table 1 and 2, respectively. The ratio of antiapoptotic to proapoptotic proteins ( $[Bcl-2]/[Bax]$ ) was calculated for each rat and particular brain region. In the amygdaloid complex, the  $[Bcl-2]/[Bax]$  decreased in the control rats subjected to trauma significantly ( $df=3$ ,  $F=41.18$ ;  $p<0.0001$ ) suggesting the presence of apoptotic changes (Fig. 2a).

Treatment of the rats with propranolol made this situation worse and no statistically significant effect of fluoxetine was observed. In the dorsal hippocampus although the statistical analysis yielded a variance ( $df=5$ ,  $F=10.41$ ;  $p=0.0007$ ), we did not observe any statistically significant change in the control rats subjected to trauma. The  $[Bcl-2]/[Bax]$  was found to be increased in traumatized controls. The effects of propranolol were detected to be sig-



**Figure 2.** The densitometric analyses obtained from photographs of western immunoblots of 3 different brain regions of rats treated with physiological saline, fluoxetine (2.5 mg/kg) or propranolol (10 mg/kg) in predator scent test produced with cat litter ( $n=4$ ). The bars with thick borders represent the rats subjected to the predator scent (dirty cat litter) and the trauma reminder (clean litter) one week later. The control rats represented by bars having thin borders were only exposed to clean litter in two sessions. The treatments continued for one week between the 2 exposure sessions. \*different as indicated,  $p < 0.05$ .

nificant (Figure 2b). In the frontal cortex we also found significant variance ( $df=6$ ,  $F=64.64$ ;  $p<0.0001$ ) and the traumatized control rats yielded significant differences between non-traumatized control rats indicating initiation of an apoptotic process. Similarly, propranolol also



**Table 1.** The densitometric measures of the Bcl-2 blots displayed in control and traumatized rats in respect to different treatments (normalized to  $\beta$ -actin density)

Brain regions	Non-traumatized			Traumatized		
	Saline	Fluoxetine	Propranolol	Saline	Fluoxetine	Propranolol
		2.5 mg/kg	10 mg/kg		2.5 mg/kg	10 mg/kg
Amygdaloid complex	0.62±0.05	0.52±0.1	0.61±0.01	0.58±0.02	0.56±0.03	0.58±0.01
Dorsal hippocampus	0.48±0.05	0.57±0.3	0.52±0.02	0.49±0.01	0.54±0.01	0.80±0.01
Frontal cortex	0.89±0.02	0.52±0.01	0.61±0.01	0.58±0.02	0.56±0.03	0.58±0.01

**Table 2.** The densitometric measures of the Bax blots displayed in control and traumatized rats in respect to different treatments (normalized to  $\beta$ -actin density)

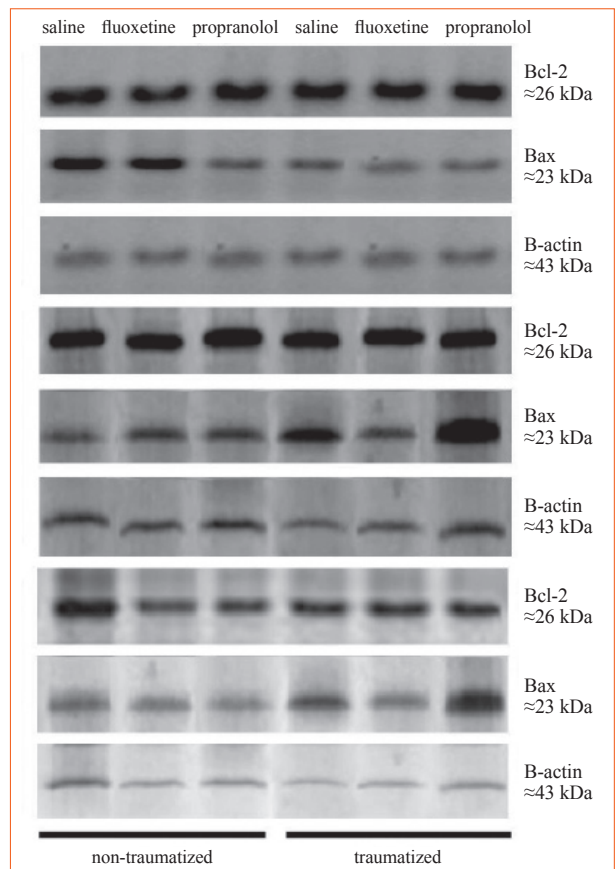
Brain regions	Non-traumatized			Traumatized		
	Saline	Fluoxetine	Propranolol	Saline	Fluoxetine	Propranolol
		2.5 mg/kg	10 mg/kg		2.5 mg/kg	10 mg/kg
Amygdaloid complex	1.23±0.01	1.21±0.1	1.41±0.02	1.41±0.01	1.22±0.01	1.60±0.01
Dorsal hippocampus	1.34±0.01	1.39±0.1	1.18±0.05	1.07±0.01	1.39±0.01	1.21±0.01
Frontal cortex	1.22±0.01	1.32±0.01	1.32±0.01	1.23±0.02	1.21±0.02	1.36±0.01

increased the apoptotic process by decreasing [Bcl-2]/[Bax] in traumatized rats in all regions. Fluoxetine treatment was not found to be different in traumatized and non-traumatized control rats in all brain regions.

Representative Western blot of Bcl-2 and Bax protein expression in rats subjected to cat litter stress treated with saline, fluoxetine or propranolol (Figure 3).

## Discussion

The present study examined the regulation of the major proapoptotic and antiapoptotic genes in predator scent test model of post-traumatic stress disorder and the efficacy of antidepressant treatments. The apoptotic process is programmed and critically controlled by a balance between proapoptotic and antiapoptotic proteins within the cell [12]. The apoptotic suppressors, Bcl-2 and Bcl-xl, interact with the death-promoting pro-apoptotic Bax in the outer membrane of mitochondria [4]. In response to stress, these proteins regulate the release of cytochrome c, which then activates caspases, the main enforcers of apoptosis. An increase in the ratio of proapoptotic vs. antiapoptotic proteins activates apoptosis and cell death occurs eventually [4]. One of the major pathways in neuronal apoptosis is the intrinsic or mitochondrial pathway. Mitochondrial damage includes rearrangement of proapoptotic and antiapoptotic molecules in its membrane. Bax exists in a stimulus-dependent equilibrium between an inactive (cytoplasmic soluble) form and an active (mitochondrial membrane associated or inserted) form that induces cytochrome c release and subsequent activation



**Figure 3.** The representative photographs of nitrocellulose membranes collected from immunoblotting experiments of rats subjected to cat litter stress treated with saline, fluoxetine or propranolol. The blots used were for Bcl-2 and Bax as indicated.  $\beta$ -actin was used to normalize the amount of protein loaded in each lane.

of the execution phase of apoptosis. Consequently, Bax could be inhibited by the anti-apoptotic Bcl-2 proteins resulting in its translocation from the mitochondrial to the cytoplasmic pool [9]. Our data showed that apoptotic changes were more pronounced in the amygdaloid complex and the frontal cortex of traumatized control rats. We need to quantify the cytochrome *c* levels to indicate that a mitochondrial pathway is involved in this model. It is long known that stressors may provoke depression-like behaviors through activation of apoptotic and antineurogenic mechanisms [25]. Magnetic resonance studies revealed that depression is accompanied by structural changes in the hippocampus, the prefrontal cortex, the amygdala, the anterior cingulate and the basal ganglia [26]. Enhanced apoptosis was detected in the brain structures of rats tested in animal models of depression, such as repeated unpredictable stress [10] and maternal separation [27]. We also demonstrated that [Bcl-2]/[Bax] ratio in the amygdaloid complex of fluoxetine treated traumatized rats was not different from the non-traumatized controls. This finding may imply the presence of a neuroprotective effect for fluoxetine. Fluoxetine, one of the selective serotonin reuptake inhibitors, shows a strong antidepressive effect and is widely used to augment the actions of serotonin in the nervous system [28-30]. The Bcl-2 and Bcl-x1 proteins have a well-known antiapoptotic activity and it was formerly demonstrated that the antidepressants upregulated Bcl-2 mRNA in rat limbic structures and frontal cortex [10]. In that study it was demonstrated that fluoxetine did not affect Bcl-2 levels but decreased Bax expression in hippocampal subregions [10]. The antiapoptotic effects of antidepressants were also confirmed by Xu et al. [31], who found an enhanced intensity of Bcl-2 immunostaining in the hippocampal mossy fibers following chronic administration of amitriptyline and venlafaxine in low (5mg/kg) but not in higher dosage (10 mg/kg) [31]. It was also demonstrated that apoptosis made a peak on the 4<sup>th</sup> day following single prolonged stress and the morphological changes were also observed in amygdala cells suggesting the critical role of amygdala in posttraumatic stress disorder [32]. These findings are also consistent with ours; we also observed apoptotic changes in the amygdaloid complex and the frontal cortex, but not in hippocampus. The most unanticipated finding of our study is the results related with propranolol. Although both of the drugs reversed behavioral parameters in traumatized rats, fluoxetine was more potent in terms of increasing antiapoptotic changes. In our experiments we observed that propranolol induced proapoptotic proteins. It is really very difficult to explain this finding but it may be used to account for the mechanism of adverse effects of propranolol, since when propranolol is used for treating hypertension, it usually produces central nervous system side effects like nightmares and depression [33,34]. Although the relation between the apoptotic changes and the affective disorders was documented, we still need new studies to affirm that

propranolol induces neuronal apoptosis. The roles of other regulatory proteins and morphological features should be examined to delineate the apoptotic effect of propranolol. It was demonstrated in pancreatic cell culture that propranolol could induce apoptosis through  $\beta_2$  adrenergic receptors [35]. The catecholamines were also shown to induce apoptosis lymphocytes [36]. The most important limitation of our study is that we only checked some of the proteins. However further studies are also required to delineate the apoptosis in this model, this work should be accepted as a pioneer work.

### **Concluding remarks**

In conclusion, the increase in apoptotic proteins was more pronounced in the amygdaloid complex and the frontal cortex in traumatized rats. The reversal of decreased ratio of [Bcl-2] and [Bax] proteins may indicate the neuroprotective effect of fluoxetine. Fluoxetine might attenuate the apoptotic pathways by activating Bcl-2 protein. The failure of propranolol in attenuating apoptosis may also account for the mechanisms of some of the adverse effects of the drug.

### **Acknowledgments**

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### **Abbreviations**

Bcl-2, the B-cell lymphoma/leukemia-2 ; PTSD, Post-traumatic stress disorder ; DEHAMER, Marmara University Animal Center; Nanxiety, the anxiety index; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonylfluoride; TBS, Tris buffer saline; TBS-T: TBS containing 0.05% Tween-20; NBT-BCIP, Nitro Blue Tetrazolium and 5-Bromo-4-Chloro-3- Indolyl Phosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

### **Conflict of Interest**

There are no conflicts of interest among the authors.

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