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Original Article

Effects of doxepin on spatial memory, TNF-α and Bcl-2 family genes expression in rat hippocampus

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Abstract

Introduction: Although the initial hypothesis for the action of doxepin was based on the inhibition of the reuptake of neurotransmitters, it has been suggested that it may also involve other mechanisms. Therefore, this study aims to investigate the effect of doxepin on spatial memory, tumor necrosis factor alpha (TNF-α) level, expression of pro-apoptotic (Bad and Bax) and anti-apoptotic (Bcl-2) genes in the rat hippocampus.

Materials and Methods: Male rats were divided randomly into three groups; the control, the doxepin 1 and 5 mg/kg, respectively). Rats received i.p injection of doxepin for 21 days. Spatial memory was evaluated by Morris water maze test. Then the hippocampi were dissected for measurement of the expression of Bcl2, Bad and Bax genes and the TNF-α level.

Results: Our results showed no significant effects of doxepin on spatial memory. Doxepin significantly decreased expression of Bad gene, but had no significant/considerable effects on Bcl2 and Bax gene expression. Also, the ratio of TNF-α to total protein (%) did not show significant differences in the rat hippocampus.

Conclusion: These results did not show any significant impact of doxepin on the factors affecting the neuronal functions in intact animals. However, Since a significant reduction in the hippocampal Bad mRNA levels was observed It is our assumption that doxepin has neuroprotective effects.

Keywords:
Doxepin;
Memory;
Hippocampus;
Bcl-2 family;
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Introduction

Although antidepressants are effective treatment for depression, their therapeutic mechanisms are not fully understood. The initial hypothesis was the enhancement of some neurotransmitters levels such as noradrenaline and serotonin. Despite the accuracy of this hypothesis, there is no explanation for a 2 to 3-week delay phase between starting of treatment and appearance of therapeutic effects. Furthermore, depletion of monoamines doesn't cause depression in healthy people (Roumestan et al., 2007; Zarei et al., 2014), therefore other mechanisms must also be involved.

Various studies on intact animals have shown that antidepressants improved cognitive processes and the expression of neuroprotective proteins, and influenced

on the development of neurons in the hippocampus (Xu et al., 2003; Kobayashi et al., 2010; Gray and Hughes, 2015). It has been demonstrated that cellular and molecular adaptations occur in brain at different levels of response to treatment with antidepressants. Antidepressants have neuroprotective effects (Jin et al., 2009). They reduce stress-induced atrophy of hippocampal CA3 pyramidal cells (Duman et al., 1999) and increase proliferation of granular cells in hippocampus (Watanabe et al., 1992). Amitriptyline can increase hippocampal brain-derived neurotrophic factor (BDNF) significantly in intact animals (Paumier et al., 2015). In addition, anti-inflammatory effects of antidepressants such as fluoxetine, clomipramine, amitriptyline and desipramine have been demonestrated (Bianchi et al., 1994; Abdel-Salam et al., 2004; Schiepers et al., 2005).

Doxepin is a tricyclic antidepressant that inhibits reuptake of norepinephrine and serotonin, and is used to treat depression and anxiety disorders (Hajak et al., 2001). BY now various properties of doxepin have been reported, such as its anti-inflammatory (Drake et al., 1994) and anticonvulsant effects (Sun et al., 2009). Furthermore, doxepin plays a protective role against oxidative stress and it increases antioxidants (Ji et al., 2004) .

Similar to other antidepressants. Doxepin may have other effects than the inhibition of reuptake of neurotransmitters and our aim was to investigate its possible effects on the various systems involved in the learning and memory. Similar to previous studies on intact animals (Xu et al., 2003; Gray and Hughes, 2015; Paumier et al., 2015), our protocols were designed to evaluate the effects of doxepin on spatial memory, tumor necrosis factor alpha (TNF-α) level, expression of pro-apoptotic (Bad and Bax) and anti-apoptotic (Bcl-2) genes in the rat hippocampus.

Materials and methods

Subjects

Thirty male Wistar rats (250-350g) were housed, five per cage and maintained on a 12 h light–dark cycle in an air conditioned constant temperature (23 ± 1°C) room, with food and water made available ad libitum. The Ethic Committee for Animal Experiments at Isfahan University approved the study and all experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. Animals were divided into three groups (n = 10 in each group): the control, the doxepin 1 mg/kg and the doxepin 5 mg/kg. Doxepin (Ray Chemicals Pvt. Ltd.) was dissolved in saline and was injected intraperitoneally. (Animals in the control group received same volume of 0.9% normal saline.)

Procedures

The rats received doxepin or an equal volume of saline daily between 7-8 am for a period of 21 days (Gharzi et al., 2012). In order to assess the changes in spatial learning and memory, the acquisition phase of Morris water maze test was conducted during 14th to 17th day, at least 3 to 4 hours after the treatment. To assess spatial memory, retention phase of the test was executed 24 hours after the last treatment (22th day). At the end of the behavioral study, rats were lightly anesthetized with diethyl ether inhalation and sacrificed by decapitation. Their brains were immediately removed from skull and both hippocampi were instantly dissected. The extracted hippocampus tissues were removed on a cold artificial CSF, and were deep frozen in liquid nitrogen, and then stored at −80°C until further studies.

Behavioral apparatus and method

Spatial learning and memory was tested by Morris water maze test. The circular tank (180 cm in diameter) was filled with water (22 ± 2 ºC) made opaque and was surrounded by a variety of extra-maze cues. The tank was divided into four quadrants, and four start positions were located at the intersection of the quadrants. Data were recorded using a camera on top of the tank and a custom software. Twenty-four hours before/prior to water maze testing, all rats were habituated to the water and the apparatus.

In the spatial acquisition phase, the rats learned to find a submerged platform using extra-maze cues. A transparent Lucite platform (10 cm) was submerged 2 cm under the water in North-east quadrant of the tank, where it remained in place for all subsequent spatial

trials. Each rat participated in 16 trials, which were organized into daily block of four trials (1 trial/start position within a block) for 4 consecutive days. For each trial, the rat was given a maximum time of 60s to locate the platform, after which it remained there for 30s. If it did not locate the platform within 60s, it was guided to it by the experimenter. The next trial started immediately after removal of rat from the platform. Escape latencies (s) were recorded (Zamani et al., 2012).

In the retention phase, one 60-sprobe trial was conducted to examine how well the rats had learned/remembered the exact location of the platform. During this trial, the platform was removed from the tank. The following measures were recorded during the probe trial: the time spent inside a circular area (70 cm diameter) around the center of platform, and platform crossing (the number of times the rat crossed the exact location of the platform).

Assessment of Bcl2, BAD and BAX levels

The brains of the rats were immediately removed from skull and the hippocampus was instantly dissected. Real-time polymerase chain reaction (Real-time PCR) was used to evaluate the expression of Bcl2, Bad and Bax genes in the half of both the hippocampi. Total RNA was extracted from hippocampus tissues using YTA kit (Yekta Tajhiz Azma, IRAN) according to the manufacturer's instructions. After isolation, quality of mRNA was checked by gel electrophoresis and RNA quantity was measured using nanodrop (OD 260nm). At the reverse transcription (RT) step, 5ng of total RNA was used to synthesis the cDNA with random hexamers primer, using the Reverta-L kit (Amplisens, Moscow) according to the manufacturer's manual.

The real-time PCR was performed using the Rotor-Gene 6000 instrument (Corbett, Life Science, Australia). SYBR Green qPCR Master Mix (2x) and specific primers (Table1) were used. 18s rRNA was used as an internal control to normalize RNA input. Cycle parameters for Real Time PCR included: 95°C for 10 min, 95°C for 15s and 60°C for 30s. The Ct value is defined as the fractional cycle number at which the fluorescence passes a fixed threshold. The fold change was calculated using the $2^{-\Delta\Delta Ct}$ method, presented as the percentage fold-expression change in treated experiment group relative to their corresponding control group after normalization to the 18srRNA endogenous control.

Assessment of TNF-α level

The other half of the hippocampi were homogenized with ice-cold 10 mMTris-HCl buffer (pH 7.4) (Lin et al., 2009).After centrifugation at 9,000 × g for 30 min at 4°C, the supernatant was collected for the measurement of total protein and TNF-α. Protein concentration was determined by a commercial protein assay, Bio-Rad RC-DC Protein assay (Bio-Rad, UK), based on the traditional method of Lowry (Lowry et al., 1951).The levels of TNF-α were measured by ELISA method using Rat TNF-α Standard ELISA Development Kit (Peprotech, USA; Cat. No.: 900-k73).

Data analysis

Data were analyzed using the SPSS 21 for Windows. The escape latencies were analyzed with two-way ANOVA followed by Tukey's post-hoc test. The probe trial data for the number of platform crossing analyzed statistically using Kruskal-Wallis Test (non-parametric ANOVA) and Dunn's Multiple Comparisons for post-test. The swim time spent inside a circular area around the center of platform and the hippocampal levels of TNF-α were analyzed by one-way ANOVA and followed by Tukey's post-hoc test. The results for gene expression analyzed with one sample t-test with the significant level set at $p<0.05$. Results are expressed as mean ± S.E.M.

Results

Morris water maze test

All rats showed a reduction in escape latencies (BLOCK effect, $F(3, 81) = 18.69$, $p<0.001$; Figure. 1) across blocks of trials, indicating spatial learning acquisition. The pattern of reduction in escape latencies to locate the platform across the blocks was same between/among the groups (Figure. 1).

The mean time spent inside a circular area (70 cm diameter) around the center of plat form (Control: 20.9±1.74s, Doxepin 1mg/kg: 18.9±2.65s, Doxepin

Table 1. Primers used in real-time PCR experiments.

Name	Sequences (5' to 3')
Bcl2-F	TAACGGAGGCTGGGATGC
Bcl2-R	TGAGCAGCGTCTTCAGAGA
Bax-F	GAGGCAGCGGCAGTGATG
Bax-R	TCCTGGATGAAACCCTGTAGCA
Bad-F	GACCAGCAGCCCAGAGTAT
Bad-R	CGCCTCCATGATGACTGTTATTG
18srRNA-F	GTT GGT TTT CGG ACC TGA GGC
18srRNA-R	GTC GGC ATC GTT TAT GGT CG

18s rRNA was used as a housekeeping gene to compare the samples.

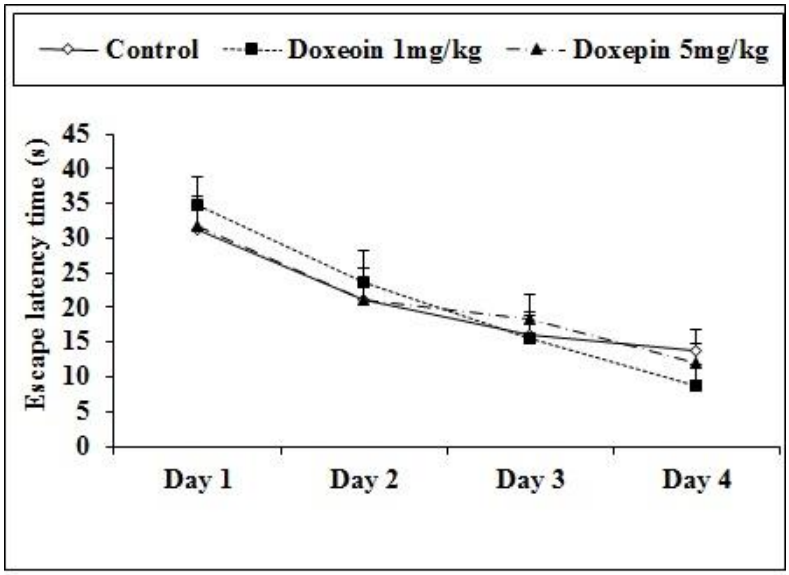

Fig.1. Effects of doxepin on the escape latencies at different blocks. Each point represents the block mean±SEM of 4 swims. Lower numbers indicate better performance (n = 10). There is no significant effect of doxepin on the escape latencies.

5mg/kg: 17.1±1.44; F (2, 29) = 0.88, p=0.42; Figure 2A) and the numbers of plat crossing (Control: 1.6±0.26, Doxepin 1mg/kg: 1.5±0.37, Doxepin 5mg/kg: 1.4±0.3; F (2, 29) = 0.09, p=0.9; Figure 2B) were used for assessment of the results of probe trial. These variables did not show significant differences between the all groups.

Gene expression of Bcl2, Bad and Bax

As seen in figure3, the mRNA expression of Bcl2, Bad and Bax genes did not show significant change in doxepin 1mg/kg compared to control group. However, the reduction in hippocampal Bad mRNA levels with respect to control group was nearly 41% (p=0.07).

Also, the mRNA expression of Bad had shown significant decreases (78%, p< 0.001) in doxepin 5mg/kg group when compared to control group (Figure 3).

TNF-α level

The various dosages s of doxepin had no significant effects on the ratio of TNF-α level to total protein in the hippocampus compared to the control group (Control: $4.64\times10^{-7}\pm1.58\times10^{-7}$, Doxepin 1mg/kg: $5.55\times10^{-7}\pm1.94\times10^{-7}$, Doxepin

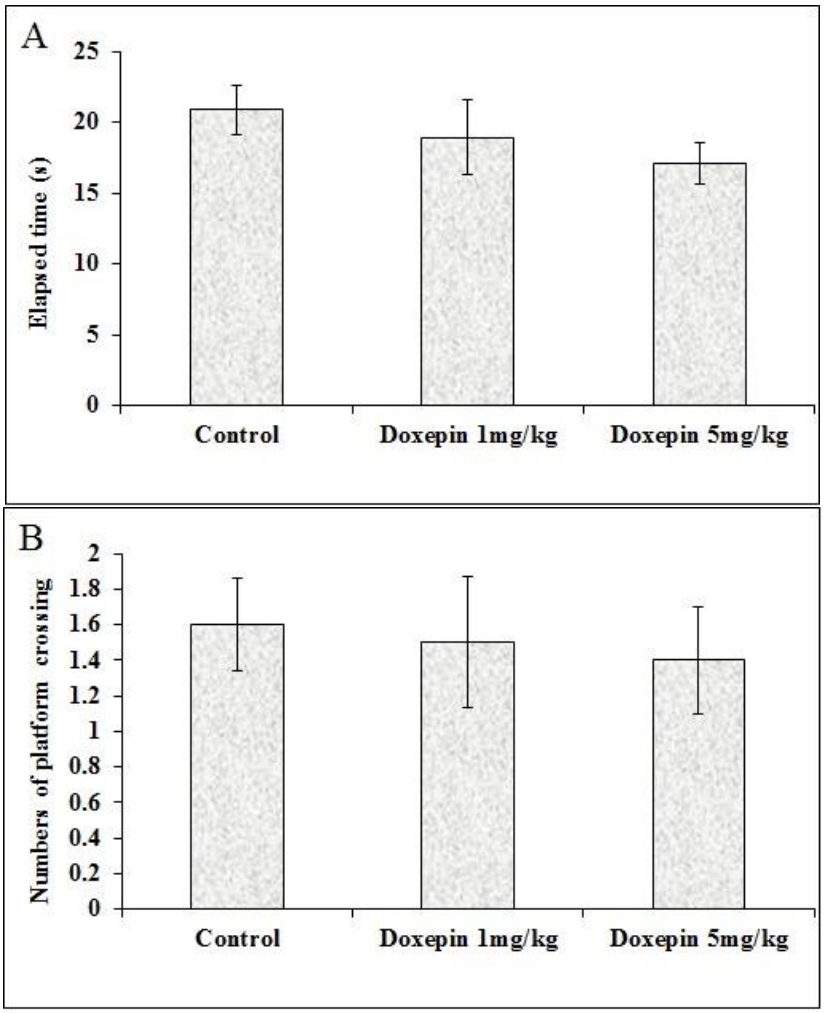

Fig. 2. Effects of doxepin on performance during the probe trial as measured by the mean time spent inside a circular (70 cm diameter) around the center of platform (A) and the number of plat crossing, 5 days after spatial acquisition phase (n=10). There is no significant difference between the groups, p>0.05.

5mg/kg: $5.93\times10^{-7}\pm2.10\times10^{-7}$; F (2, 23) = 0.12, p=0.88; Figure 4).

Discussion

The present findings showed that doxepin decreased hippocampal expression of Bad gene, whereas it had no detectable effects on the expression of BCl2 and Bax genes (Figure 3).

Previous studies have shown that although low doses of antidepressants, such as amitriptyline and venlafaxine increases Bcl-2 expression in hippocampal neurons of intact rats, in high doses they have no effect on Bcl-2 expression (Xu et al., 2003). Thus, the effects of antidepressants, such as doxepin may be different at low and high doses. It has been demonstrated that chronic administration of duloxetine and mirtazapine decreases proapoptotic proteins such as Bad and bax, and increases neurotrophin gene expression such as Bcl-2 in the hippocampus and cerebral cortex of intact rats (Engel et al., 2013). But these effects were not the same in other brain areas and were partially dependent on time (Paumier et al., 2015). In line with these studies, our results indicates that the pro-apoptotic and anti-apoptotic genes are involved in the actions of antidepressants. But the effects of antidepressants on apoptosis are complex and presumably depend on antidepressant type (Djordjevic et al., 2012).

Because doxepin can change gene functions within the hippocampus, it may affect learning and memory (Lee et al., 2013). However, we observed that doxepin had no significant effects on the spatial learning and memory. In the previous study, we observed that

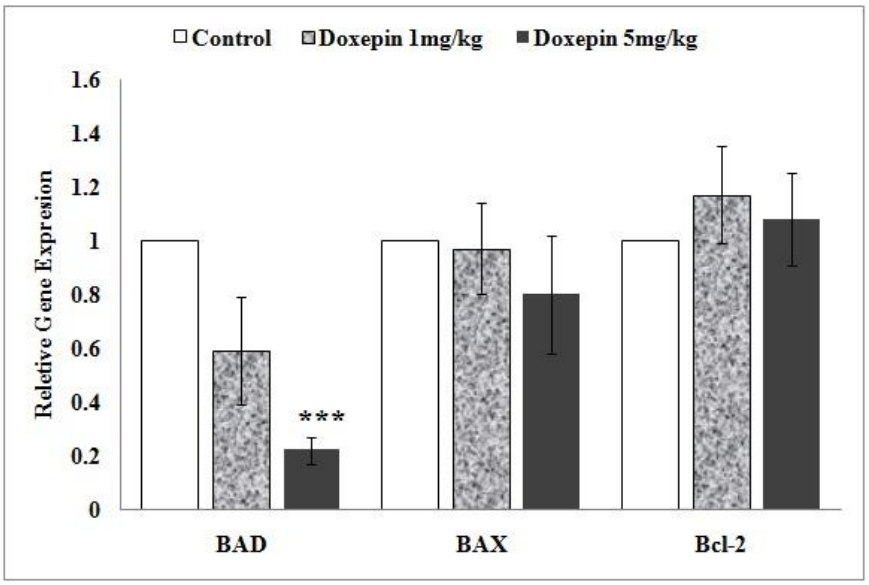

Fig. 3. Effects of doxepin on relative gene expression of pro-apoptotic (Bad and Bax) and anti-apoptotic (Bcl-2) genes, the amount of expression was measured by RT-PCR. The mRNA expression data normalized to the 18srRNA signal. Fold changes relative to control are presented. Mean ± SEM values of experiments are shown. ***p < 0.001 with respect to the control group (n=7).

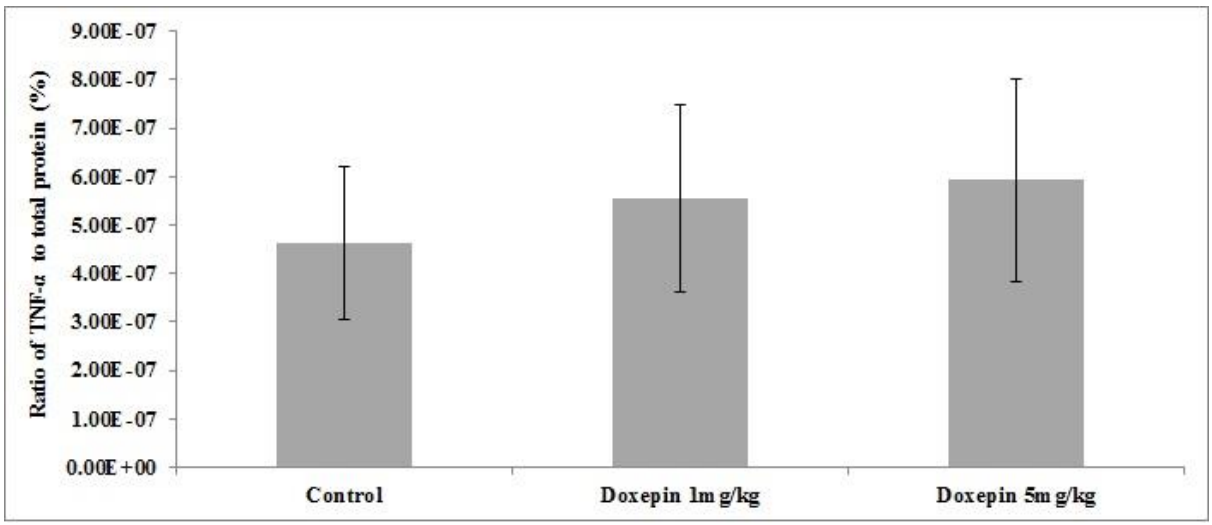

Fig. 4. Effects of doxepin on ratio of TNF-α to total protein (%) in the hippocampus. Data are expressed as mean ± SEM. (n = 8). There is no significant difference between the groups, p>0.05.

chronic administration of doxepin in intact rats improves the passive avoidance learning (Gharzi et al., 2012), but in this study there was no significant change in the spatial memory. These results could be due to the complexity and type of tests and the different mechanisms involved. Also, the timing of protocols for the two studies have been different. In the previous study, the acquisition phase of passive avoidance learning happened at the end of the twenty-one-day period of treatment and one day after that, the retention phase was performed. But in the present study acquisition phase of Morris water maze test run occurred during the fourteenth to the seventeenth day of the twenty-one-day period of treatment, and spatial memory assessment was done on the day after the last injection of doxepin, therefore, in this study we looked at and measured memory on a longer scale.

It is worth mentioning that gene expression does not always cause protein production and other conditions such as the length of time are effective in developing a response. Studies have shown that the use of antidepressants have different effects in the long term

than in the short term. For example, it has been shown that the use of amitriptyline for 3 weeks damage the learning process (Richardson et al., 1994). However, other study has shown that administration of amitriptyline for two months facilitated hippocampal (spatial) learning and memory in young rats (Yau et al., 2002). Therefore, it seems that the effects of antidepressants are probably time- dependent.

Additionally , we observed that doxepin had no significant effects on the level of hippocampal TNF-α, whereas, other reports demonstrated the anti-inflammatory effects of doxepin in peripheral tissues (Drake et al., 1994; Ji et al., 2004). Since in this study doxepin was used in intact animals that had no prior history of inflammation, the TNF-α level was unchanged in the rats. However, these results do not rule out the possibility that doxepin leads to a reduction in the inflammatory process in the nervous tissue, if there is an inflammation in the nervous system.

In conclusion, our findings suggest that doxepin affects the gene functions in the hippocampus. The data suggest/point to the possibility that doxepin may have neuroprotective effects, however, further studies are needed to understand the effects of doxepin.

Acknowledgments

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Conflict of interest

The authors declare that they don't have any conflict of interest.

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