



Research Article

Coumestrol Alleviates Oxidative Stress, Apoptosis and Cognitive Impairments Through Hippocampal Estrogen Receptor-Beta in Male Mouse Model of Chronic Restraint Stress

Kiarash Fekri^{1,2}, Javad Mahmoudi¹, Saeed Sadigh-Eteghad¹, Fereshteh Farajdokht¹, Alireza Mohajjel Nayebi^{2,3*}

¹Neurosciences Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

²Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.

³Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

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Abstract

Background: Coumestrol is well-known as a natural estrogen receptor-beta modulator. Since the role of estrogen receptors in controlling stressful situations has already been reported and their cognitive functions in hippocampus seem to be independent of sexual tasks, the aim of this study was to investigate the improving effects of this phytoestrogen on negative consequences of exposing male mice to chronic restraint stress.

Methods: This study was divided into two separate but consecutive phases. In the first phase, the possible effects of coumestrol (30, 60, 120 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, i.p.) and its vehicle (sesame oil) on restraint stress-induced cognitive impairments, oxidative stress and apoptosis were evaluated. During the second phase, a selective estrogen receptor-beta antagonist was used to investigate the possible involvement of beta-type estrogen receptors in these processes. Morris water maze and novel object recognition tests were performed to evaluate memory while elevated plus maze and open field tests were used to measure the level of anxiety. Spectroscopy and western blotting methods were also employed to evaluate oxidative and apoptotic status in hippocampal tissues. Furthermore, serum level of corticosterone was measured for each group.

Results: Behavioral tests indicated memory enhancing and anxiolytic effects of coumestrol. Biochemical evaluations also proved its antioxidant and anti-apoptotic potential. On the other hand, the mentioned behavioral and biochemical improvements were reversed in the group treated with estrogen receptor-beta antagonist.

Conclusion: Coumestrol may ameliorate negative consequences of exposure to chronic stress such as oxidative stress, apoptosis and cognitive impairments, via the modulation of beta-type estrogen receptors in hippocampus.

Introduction

Stress is a natural response of the body that occurs as a result of confrontation with a stimulus in a way that a wide range of intrinsic or extrinsic stimuli can trigger this biological reaction. Although it is necessary for coping with challenging situations, stress has both positive and negative consequences and along with the lifesaving effects it may lead to severe health damages.¹⁻⁴

Among multiple systems underlying stress, hypothalamic-pituitary-adrenal (HPA) axis has been known to be almost the most important. This axis, which its activation leads to glucocorticoid release, is modulated through negative feedback loops and stimulatory signals.⁵

Regardless of its mechanism, stress has been known as one of the initiating factors for the development of various neurodegenerative conditions and it would cause

structural and functional alterations in different regions of brain. For instance, hippocampus which is one of the important regions in brain and plays key roles in regulating mood, memory and cognition, would be highly affected by different types of stress.⁶ Studies have shown that tolerating stressful situations in early years of life, would result in a smaller hippocampus at older ages. The outcomes of this resizing can be observed in some behavioral tests such as water mazes. Although facing short-term stressors improves cognitive functions temporarily, chronic conditions can disrupt learning and memory. However, defining an exact correlation between stress and cognition seems to be difficult. The obvious fact is that stress changes the learning procedure and impairs memory significantly.^{7,8}

Reports indicate significant roles for oxidative stress and apoptosis in the development of depressive-like behaviors

*Corresponding Author: Alireza Mohajjel Nayebi, E-mail: nayebia@tbzmed.ac.ir

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following facing stressful conditions.⁹ Based on this surmise, many antioxidant compounds with neuroprotective capacity have been evaluated for their possible attenuating effects on the destructive consequences of enduring chronic stress such as anxiety and depression.¹⁰⁻¹³ On the other hand, sex hormones seem to be strongly involved in regulating the responses to stressful situations. Therefore, the relationship between these hormones, stress and hippocampal function have been investigated in numerous studies.¹⁴⁻¹⁶

Lots of in-vivo models have been developed to investigate possible pharmacological solutions against the destructive consequences of tolerating stressful conditions. Among them predator stress, neonatal isolation, circadian rhythm changes and restraint stress can be mentioned.¹⁷⁻²⁰ Particularly, restraint stress has been known as a widely used model which can evaluate different treatments for negative consequences of facing stressors in recent decades.²¹⁻²⁴

Estrogens are a group of endogenous hormones that help control stress-related behaviors as well as reproductive functions. They can act with or without interaction with receptors. It has been suggested that estrogens' effects on cognition would be mainly modulated by binding to estrogen receptor-beta (ER β). According to previous studies, hippocampal ER β s are expressed in both males and females and their functions are independent of sexual tasks and mostly related to memory and cognition.²⁵⁻²⁷

Possible estrogenic activity of herbs (phytoestrogens) has been studied widely since the 1940s. In this regard, lots of studies showed the cognitive benefits of phytoestrogens.²⁸⁻³⁰ Coumestrol (COUM) which belongs to a natural group of phenols is a phytoestrogen that is found abundantly in alfalfa and exerts various pharmacological effects throughout the body.³¹⁻³³ Apart from affecting sexual function and behaviors, the phytoestrogen is a powerful antioxidant and has shown to cause notable neurological alterations which most of them seem to be mediated through hippocampal ER β s.³⁴⁻³⁷ It has been shown that COUM would be able to protect neurons against neonatal hypoxia-ischemia. Also the possible role of ER β s in developing its neuroprotective properties has been discussed.³⁸ Furthermore, according to a previous report, COUM would attenuate the damages to astrocytes via possible interactions with ER β s while being exposed to β -amyloid peptide and lipopolysaccharide.³⁹

These records suggest the potential ability of COUM in alleviating the negative consequences of facing stressful situations probably through modulation of hippocampal ER β s. Furthermore as already noted, functions of the mentioned receptors do not differ between the two sexes. So based on this background, we aimed to further investigate the possible effects of COUM on oxidative stress, apoptosis and cognitive impairments in male mouse model of chronic restraint stress.

Materials and Methods

Animals

Ninety-one male BALB/c mice (8-10 weeks old weighing 20-30 g) were prepared from Tabriz University of Medical Sciences and maintained in standard polypropylene cages at 22-25 °C under a 12:12 h light/dark cycle with access to food and water *ad libitum*. All experiments and procedures were performed between 09:00 and 14:00, in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee for the Use of Animals in Research at Tabriz University of Medical Sciences (Code:IR.TBZMED.VCR.REC.1397.245).

Chemicals

COUM was purchased from Carbosynth (UK) and 0.2% solution of dimethyl sulfoxide (DMSO) in sesame oil (one of the previously used vehicles for COUM⁴⁰) was used as a vehicle to dissolve it. PHTPP, as the antagonist for ER β , was prepared from Sigma Aldrich Chemical Company (Germany) and dissolved in its vehicle (1% solution of DMSO in normal saline).

Treatments

This study was conducted in two distinct phases which each of them consisted of five consecutive stages (Figure 1), described completely in the following sections. In the first phase, which was done to determine the effective dose of COUM, mice (n=65) were divided into five groups (n=13 in each) as Control, Vehicle (Veh), COUM 30, COUM 60, and COUM 120. Mice in control group did not subjected to restraint stress but were given intraperitoneal (i.p.) injections of COUM's vehicle. Along with induction of stress, animals in Veh, COUM 30, COUM 60, and COUM 120 groups received i.p. injections of COUM's vehicle (10 ml.kg⁻¹.day⁻¹), 30, 60, and 120 μ g.kg⁻¹.day⁻¹ of COUM, respectively. Based on the results obtained from the first phase, the dose that was able to alleviate oxidative stress, apoptosis and cognitive impairments the most, was considered as the effective dose.

The second phase was done to evaluate the possible contribution of hippocampal ER β s in forming behavioral and neurochemical effects of COUM. In this phase, mice (n=26) were randomly divided into two groups (n=13 in each) and stressed (using the same protocol employed in the first phase). In addition to tolerating stress, one of the groups, received the effective daily doses of COUM (i.p.) as well as subcutaneous (s.c.) injections of the vehicle for PHTPP while the other received the doses of COUM (i.p.) and PHTPP (0.8 mg.kg⁻¹.day⁻¹, s.c.). Chemicals were prepared freshly before administration and injected at a constant volume of 10 ml.kg⁻¹ body weight.



Figure 1. The order of the stages in each phase.

Restraint stress

In order to induce chronic stress, the mice were immobilized 2 hours a day (from 9 to 11 in the morning) by being placed into well-ventilated 50 ml falcon tubes (with 12 holes) for 14 consecutive days.⁶

Behavioral tests

Behavioral tests were performed one by one, 24 hours after completing the treatment protocol. Notably all the devices used in this study, were purchased from Arman Poshtiban Teb Co (Tabriz, Iran).

Open field test

To perform the test, an open field (OF) arena (33×33×33 cm) made of opaque black polyethylene was used. One by one, the mice were gently placed at the center of the box where over a time interval of 5 minutes, the activity of the animals were monitored and the considered behavioral parameters (the total distance traveled and the time spent in the central area) were recorded.^{1,41}

Elevated plus maze test

In order to perform elevated plus maze (EPM) test, each animal was carefully placed on the central area of an apparatus consisted of two opposite open arms (30 × 5 cm and 0.5 cm edge) and two opposite closed arms (30 × 5 cm and 15 cm high wall) which were elevated 50 cm from the ground. After a time interval of 30 seconds (which was considered for habituation), the movements of each mouse was monitored for 5 minutes and the time spent in the open arms was recorded.⁴²

Novel object recognition test

One of the most common ways to measure episodic-like memory in rodents is the novel object recognition (NOR) test.⁴³ In the current study, the test was conducted in three phases (in three consecutive days) including habituation, training and retention using an open-field box (33×33×33 cm) and some ordinary objects (odorless and hard to chew). In the habituation phase, the mice were placed in the empty box one by one for 5 minutes. In the training phase, each mouse was exposed to two identical objects which were tightly fixed inside the box and video-tracking took place for 5 minutes. During the last phase, one of the objects was replaced with a distinct one, the movements of the mice were monitored for 5 minutes and discrimination index (DI) was calculated for each animal as follows: $DI = (N-F) / (N+F)$. In the mentioned equation, N stands for the time the mice spent around the new object and F stands for the time they spent to explore the familiar one.⁴⁴

Morris water maze test

The Morris water maze (MWM) test was used to evaluate spatial memory. In order to perform the test in this research, a black round pool (75 cm in diameter and 35 cm in height with an escape platform inside) was filled with water (24-25°C). The test was conducted in three consecutive phases

of visible platform, hidden platform and probe trial. The first phase (visible platform) started with virtually dividing the pool into four equal regions and continued by placing the platform (its surface was 1 cm higher than the water level and marked with a flag) into a quadrant and releasing the mice in turn from four different directions. For further analyses video-tracking was performed until the moment each animal found the platform (if the platform could not be found throughout 60 seconds, the mouse was led toward it manually). The second phase (hidden platform) began the next day. In this phase, the platform was unmarked and its surface was set 1 cm lower than the water level. Similar to the previous phase, each mouse was released from four directions and given 60 seconds to find the platform. The whole procedure was recorded by the camera and repeated for five consecutive days. The third phase (probe trial) was conducted in the last day by removing the platform and letting the animals (one by one) to swim in the pool for a single minute while being tracked by the camera. The escape latency times and the time the mice swam in the platform quadrant (in the last phase) were considered as the required parameters.⁴⁵

Behavioral analysis

All the recorded clips were analyzed by fully automated EthoVision XT video tracking software (Noldus, The Netherlands). Regarding the OF, EPM and NOR tests, the apparatus was cleaned with 10% solution of ethanol to eliminate the odors and olfactory cues after testing each animal. Furthermore, the water of the tank which was used in MWM test was changed daily with fresh water of the same temperature.

Biochemical analyses

Twenty-four hours after completing the behavioral tests, blood and brain (hippocampus) tissue samples were collected by sacrificing the animals following a deep anesthesia with ketamine and xylazine (90/10 mg.kg⁻¹, i.p., respectively). Taken blood specimens were placed in room temperature for 20 minutes to be stabilized and then serum samples were obtained by centrifuging (4000 rpm for 10 min at 4°C). After that, they were stored at -70 °C along with the hippocampus tissues which were separated in cold normal saline for further analyses. To avoid obtaining false results (particularly regarding corticosterone which its serum levels is significantly different between the diurnal and nocturnal phases), the whole samples were prepared between 10 and 12 in the morning.

Serum levels of corticosterone

Serum levels of corticosterone (CORT) were measured by the enzyme-linked immunosorbent assay (ELISA) kit (Abcam, ab108821, UK) based on the protocol provided by the manufacturer.

Oxidative and anti-oxidative factors

The hippocampal samples were homogenized in 1.15%

potassium chloride (KCl) solution by a tissue homogenizer. Then the samples were centrifuged (10000 rpm for 10 min at 4 °C) and the supernatants were collected. The concentration of total protein was measured using the Bradford method.

A RANSOD (Randox Laboratories Ltd, Crumlin, United Kingdom) laboratory kit was employed for measuring superoxide dismutase (SOD) activity. The absorbance was measured spectrophotometrically (Shimadzu Corporation) at 505 nm and the results were expressed as U/mg protein.

A RANSEL (Randox Laboratories Ltd, Crumlin, United Kingdom) laboratory kit was employed for measuring glutathione peroxidase (GPx) activity. The method was based on a reaction in which oxidized glutathione is reduced back rapidly to glutathione in the presence of NADPH and glutathione reductase. A spectrophotometer (Shimadzu Corporation) was used for reading the absorbance (at 340 nm) and GPx concentration was presented as U/mg protein.

Thiobarbituric acid reaction (TBAR) colorimetric assay was employed for measuring malondialdehyde (MDA) levels in which, optical density of the supernatant was recorded following being evaluated by a plate reader (at 532 nm, Shimadzu Corporation) and presented as nmol/mg protein.

A Randox total antioxidant status kit (Randox Laboratories Ltd, Crumlin, United Kingdom) was employed for evaluating total antioxidant capacity (TAC) based on the 2'-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS^{•+}) method. A spectrophotometer (Shimadzu Corporation) was used for reading the absorbance (which was presented as $\mu\text{mol}/\text{mg}$ protein) at 600 nm.

Western blotting

The levels of apoptotic and anti-apoptotic proteins were evaluated by western blotting method. After lysing, homogenizing and centrifuging the tissues, the supernatants were collected and total protein concentrations were calculated according to Bradford method. For this purpose, protease inhibitor cocktail was used along with Radio Immuno Precipitation Assay (RIPA) lysis buffer (pH=8.0) which consisted of Tris, Triton X-100, sodium chloride, sodium dodecyl sulfate and sodium deoxycholate.

SDS-polyacrylamide gel electrophoresis was employed for separating the protein samples into 20 μg portions. Then they were placed on a polyvinylidene difluoride (PVDF) membrane (Roche, UK) and non-specific binding reactions were inhibited by incubating the membranes with a blocking solution for 2 h at room temperature. The blocking solution consisted of bovine serum albumin (BSA) 3% in Tris-buffered saline (pH 7.5). During the night, the membranes were incubated with rabbit primary antibodies; anti- β -actin (as the internal control of cytosolic proteins, sc-47778), anti-Bcl-2 (sc-7382), anti-BAX (sc-70405), anti-caspase-3 (sc-136219) and anti-caspase-9 (sc-81663). The whole antibodies were prepared from

Santa Cruz Biotechnology, Inc. (Texas, USA). Then the membranes were washed (3 times) with Phosphate Buffered Saline (PBS) and incubated with horseradish peroxidase-conjugated (HRP) goat anti-rabbit IgG secondary antibody for 2 h at room temperature. In the last step, the protein bands (Amersham, UK) were visualized using an enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL) and Image J 1.62 software was employed for quantifying their relative optical density.

Statistical analysis

Each data set was statistically analyzed by GraphPad Prism 8 software. The data were expressed as the mean \pm SEM and were compared using the independent-samples t-test as well as one-way and two-way analysis of variance (ANOVA) followed by the multiple comparison test of Tukey. Differences between the means were considered statistically significant if $p < 0.05$.

Results

Effects of COUM on behavioral and biochemical factors OF test

The data represented no significant differences ($p > 0.05$) regarding locomotor activity between the studied groups (Figure 2A) while the time intervals spent in center were meaningfully ($p < 0.001$) longer in COUM ($120 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$)-treated mice (Figure 2B).

EPM test

The results showed significant differences in the time intervals the mice spent in the open arms between vehicle- and COUM-treated groups (Figure 2C). As shown in the figure, the changes were dose-dependent with $p < 0.05$ and $p < 0.01$ in COUM 60 and COUM 120 groups respectively.

MWM test

The first phase (visible platform), showed no significant differences in terms of the mean swimming speed and the total distance traveled between the groups (the data are not presented). Consecutively, according to the results obtained from the second stage (hidden platform), the latency times (which were longer in Veh group when compared to control) in COUM-receiving groups became significantly shorter in comparison with the times recorded for Veh group (Figure 3A). Finally, data analysis of the last stage showed a significant increase in the average time the COUM-receiving mice spent in target quadrant when compared to that observed for Veh-treated group (Figure 3B).

NOR test

According to Figure 3C, pharmacological treatment of the mice with COUM (60 and $120 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) resulted in a significant increase ($p < 0.01$) in the average DI.

CORT

As one of the most important indicators for stress in

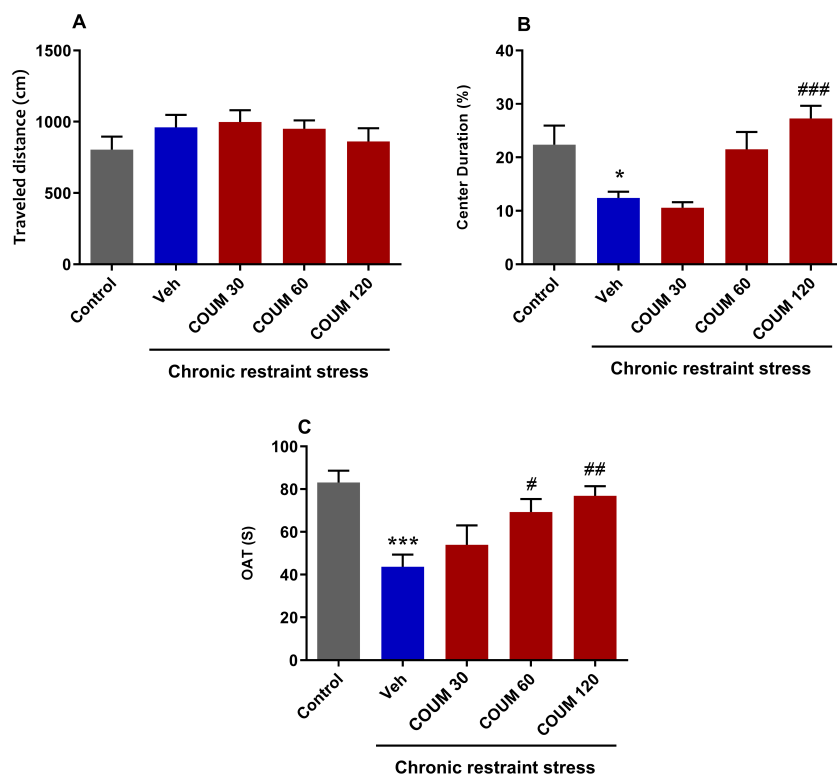


Figure 2. Effects of COUM on anxiety-like behaviors. A and B: OF test, C: EPM test. n = 13 in each group. * $p < 0.05$ and *** $p < 0.001$ compared to control group, # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ compared to Veh group. OAT: Open arm time, Veh: Vehicle, COUM: Coumestrol.

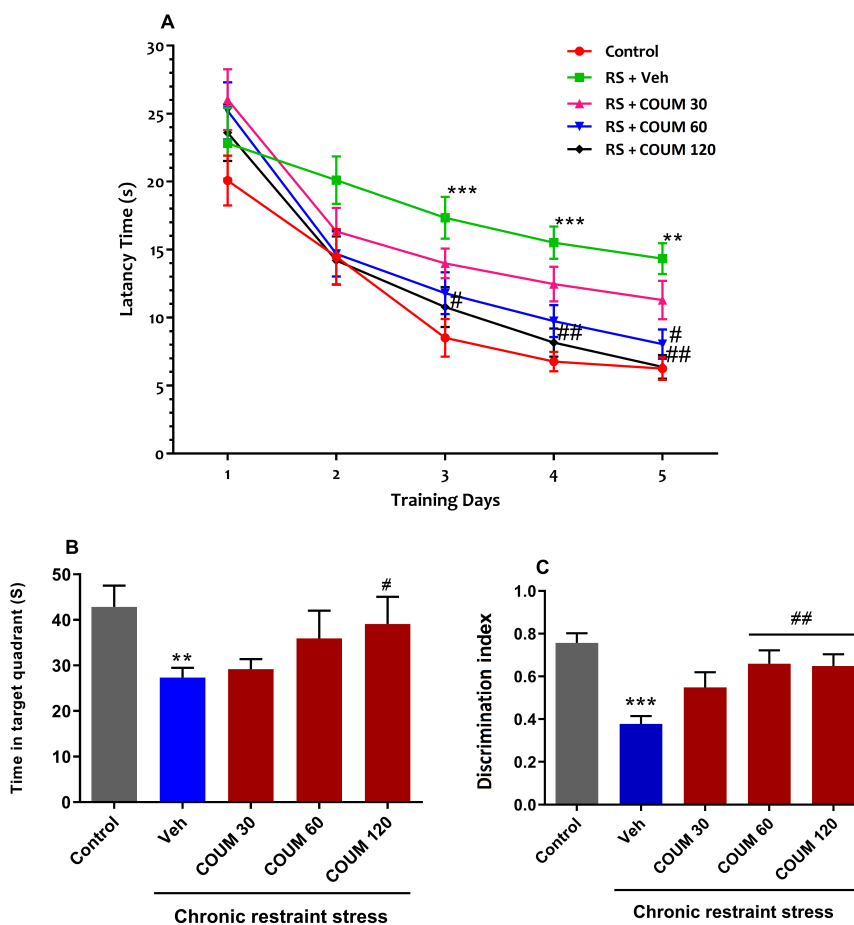


Figure 3. Effects of COUM on memory impairments. A and B: MWM test, C: NOR test. n = 13 in each group. ** $p < 0.01$ and *** $p < 0.001$ compared to control group, # $p < 0.05$ and ## $p < 0.01$ compared to Veh group. RS: Restraint stress, Veh: Vehicle, COUM: Coumestrol.

rodents, CORT levels were monitored in all groups. According to Figure 4, COUM administration ($120 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, i.p.) clearly attenuated the increase in CORT levels that had occurred as a result of stress induction.

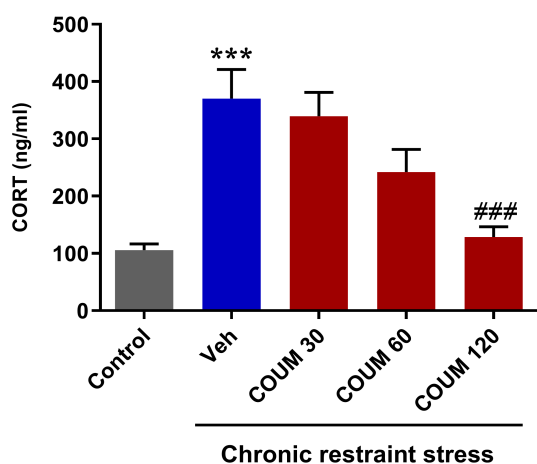


Figure 4. Effects of coumestrol on CORT levels. $n = 6$ in each group. *** $p < 0.001$ compared to control group and ### $p < 0.001$ compared to Veh group. CORT: Corticosterone, Veh: Vehicle, COUM: Coumestrol.

Oxidative and anti-oxidative factors

Significant differences between the groups were also observed regarding oxidative and anti-oxidative factors. As can be seen in Figure 5, MDA levels which had been elevated in vehicle-receiving mice, were closer to normal status in COUM-treated groups (in a dose-dependent manner). On the other hand, in Veh group, GPx, SOD and TAC levels were significantly lower compared to control while the decreases were compensated in the mice treated with COUM.

Apoptotic and anti-apoptotic factors

Chronic stress increased apoptotic (BAX and caspases) and decreased anti-apoptotic (Bcl-2) factors in Veh group while the changes were alleviated in the mice treated with COUM (Figure 6).

Administration of ER β antagonist

Based on the results of the first phase, the highest studied dose of COUM ($120 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, i.p.) was selected as the most effective one. To investigate the possible role of ER β , two new groups were treated with this dose of COUM while being exposed to chronic stress. The difference between the

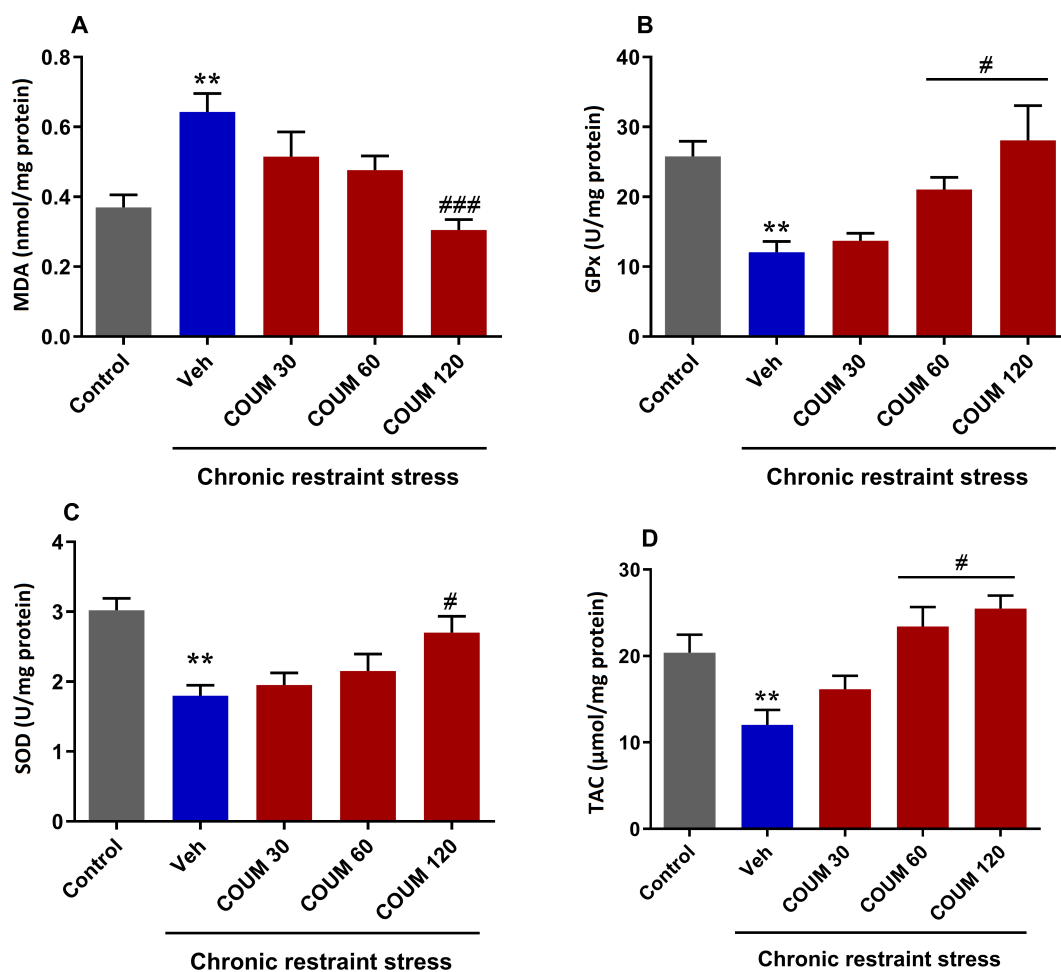


Figure 5. Effects of COUM on oxidative and anti-oxidative factors. A: MDA, B: GPx, C: SOD, D: TAC. $n = 6$ in each group. ** $p < 0.01$ compared to control group, ### $p < 0.001$ and # $p < 0.05$ compared to Veh group. MDA: Malondialdehyde, GPx: Glutathione peroxidase, SOD: superoxide dismutase, TAC: Total antioxidant capacity, Veh: Vehicle, COUM: Coumestrol.

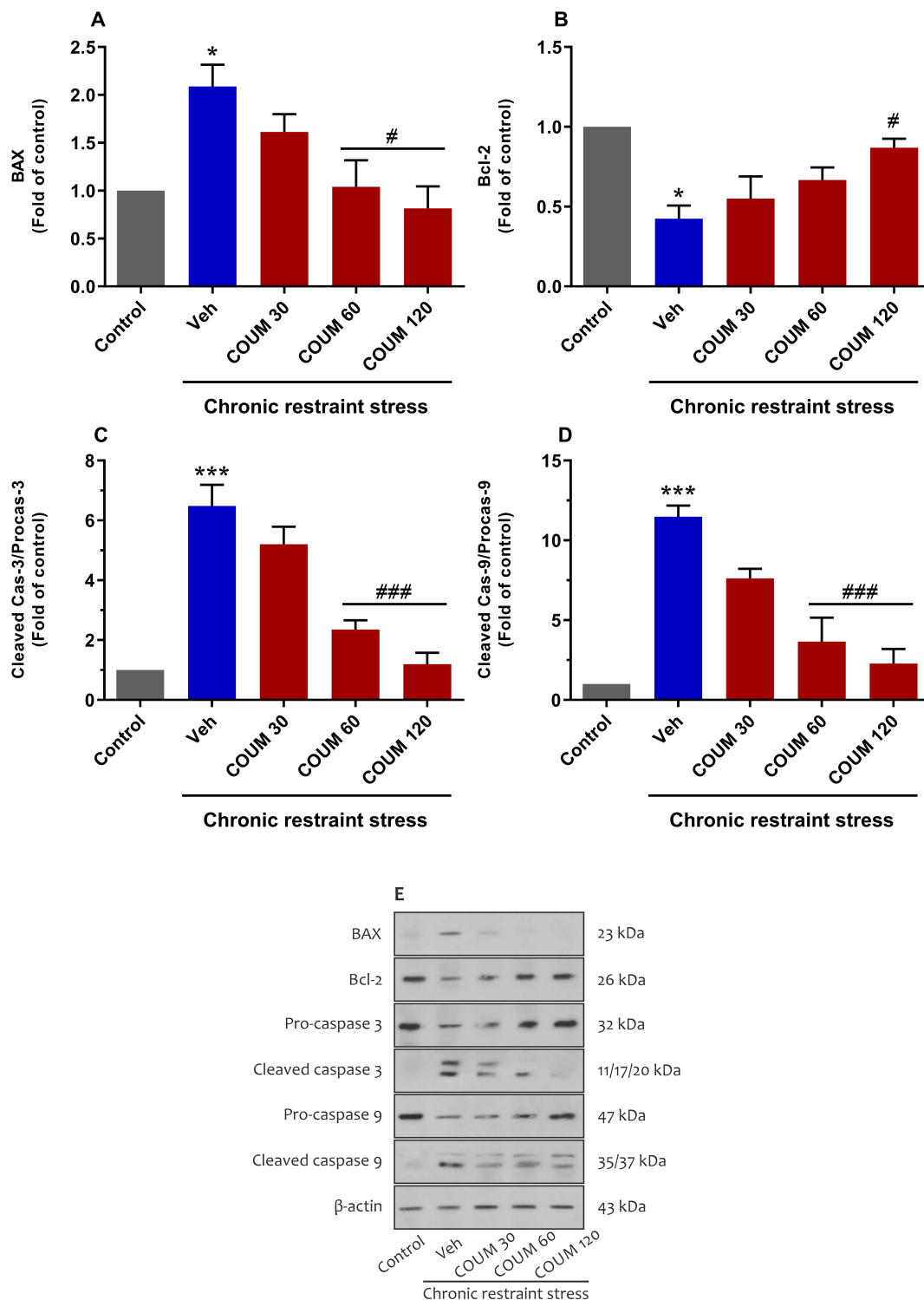


Figure 6. Effects of COUM on apoptotic and anti-apoptotic factors. A: BAX, B: Bcl-2, C: Caspase-3, D: Caspase-9, E: Image of correspond blots. n = 3 in each group. * $p < 0.05$ and *** $p < 0.001$ compared to control group, # $p < 0.05$ and ### $p < 0.001$ compared to Veh group. BAX: Bcl-2 associated X protein, Veh: Vehicle, COUM: Coumestrol.

two was that one received PHTPP ($0.8 \text{ mg.kg}^{-1}.\text{day}^{-1}$, s.c.) in parallel with COUM but the other received the vehicle for PHTPP.

Statistical analysis of the data showed that administration of the antagonist would eliminate the anxiolytic effects of COUM. According to Figure 7, treating the animals with

PHTPP reduced the time spent in the central square and open arms in OF and EPM tests respectively (while making no changes to locomotor activity as expected).

The changes which were made to memory indicators in the antagonist-treated group can be found through Figure 8. During the first stage (visible platform) of MWM test,

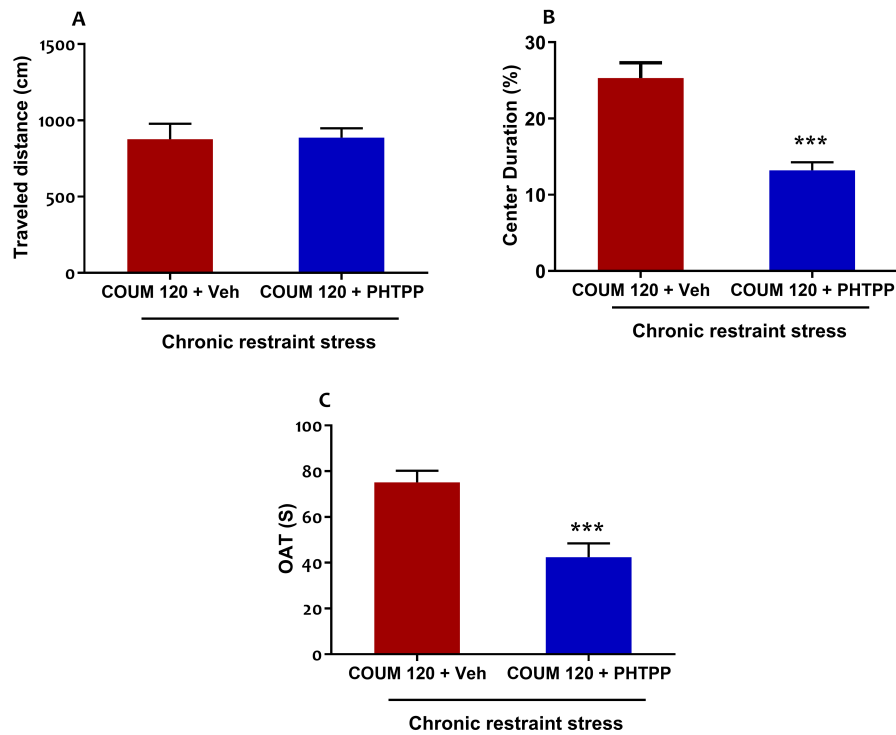


Figure 7. Effects of antagonist administration on anxiolytic effects of COUM. A and B: OF test, C: EPM test. n = 13 in each group. *** $p < 0.001$ compared to COUM 120 group. OAT: Open arm time, COUM: Coumestrol, Veh: Vehicle for PHTPP.

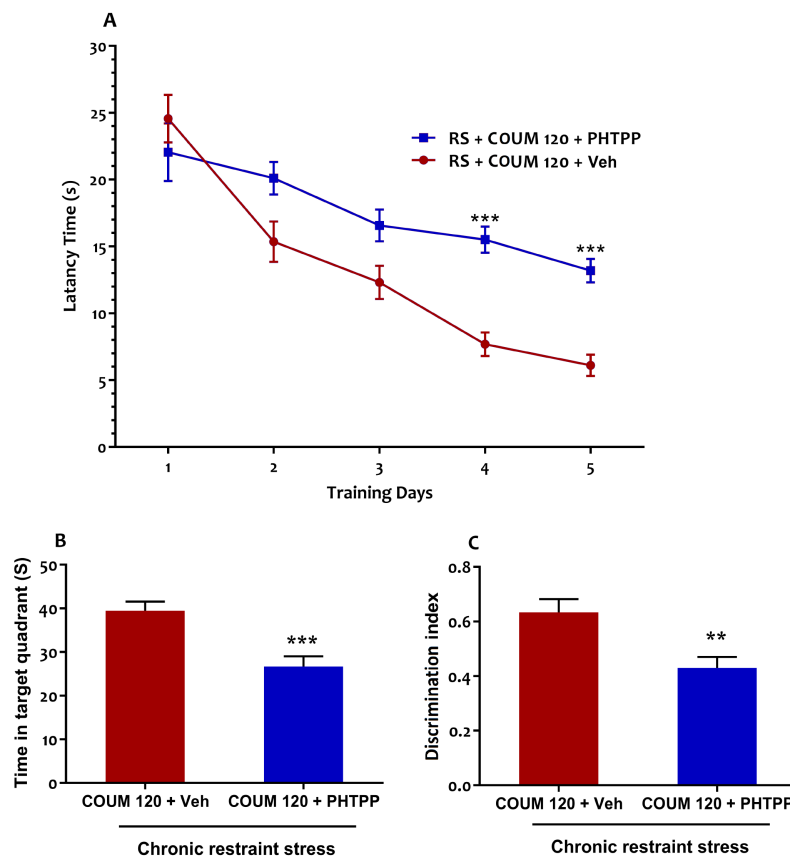


Figure 8. Effects of antagonist administration on memory-enhancing effects of coumestrol. A and B: MWM test, C: NOR test. n = 13 in each group. ** $p < 0.01$ and *** $p < 0.001$ compared to COUM 120 group. RS: Restraint stress, COUM: Coumestrol, Veh: Vehicle for PHTPP.

there were no significant differences in terms of the mean swimming speed and the total distance traveled between the two groups, as expected (the data are not presented).

Afterwards, according to the results obtained from the second stage (hidden platform), the latency times in PHTPP-receiving mice were significantly longer in comparison with the times recorded for COUM-treated group (Figure 8A). Finally, data analysis of the last stage showed a significant decrease in the average time the antagonist-receiving mice spent in target quadrant when compared to that observed for the mice treated with the effective dose of COUM (Figure 8B).

On the other hand, considering the results of the NOR test (Figure 8C), pharmacological treatment of the mice with the antagonist, significantly ($p < 0.01$) attenuated the elevation that was made to average DI in COUM-treated group.

Biochemical evaluations also showed the reversal effects following the antagonist administration. As shown in Figure 9, CORT levels were higher in PHTPP-treated group. Besides, administering PHTPP (in antagonist-treated group) attenuated the anti-oxidative effects of COUM, so that the changes in MDA, GPx and TAC levels were statistically significant (Figure 10).

Furthermore, data analyses showed remarkable deterioration in the whole apoptotic and anti-apoptotic markers in antagonist-treated group. According to the results obtained from western blotting, the decreases in hippocampal amounts of BAX and caspases (as well as the

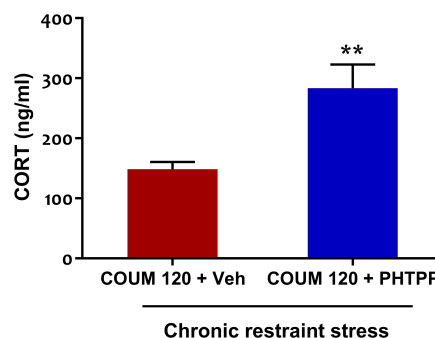


Figure 9. Effects of antagonist administration on plasma levels of CORT. $n = 6$ in each group. ** $p < 0.01$ compared to COUM 120 group. CORT: Corticosterone, COUM: Coumestrol, Veh: Vehicle for PHTPP.

rises in Bcl-2 levels) were reversed in PHTPP-receiving mice (Figure 11).

Discussion

The results showed that systemic administration of COUM would be able to alleviate oxidative stress, apoptosis and behavioral impairments in male mouse model of chronic restraint stress. On the other hand, the second phase of this study indicated the involvement of ER β s in development process of the mentioned effects.

As already mentioned, stress can be induced in laboratory animals through various methods. Exposure to a cat (for a rodent), isolating the neonates, changing the circadian rhythm, exposure to a noisy stimulus, changing

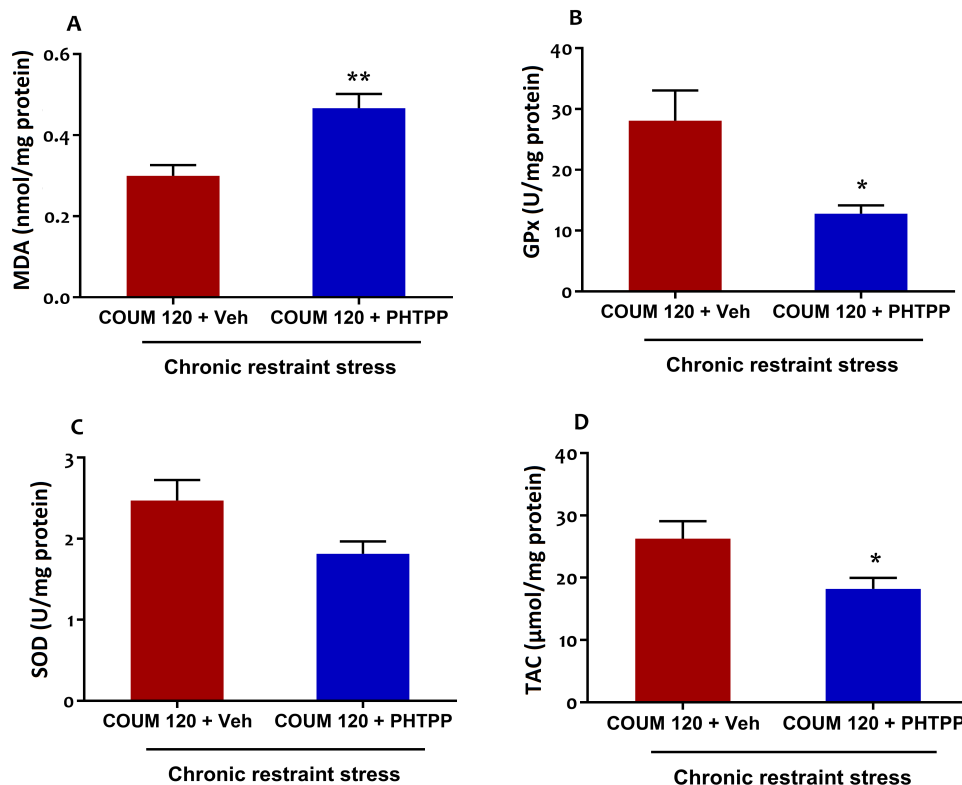


Figure 10. Effects of antagonist administration on oxidative and anti-oxidative parameters. $n = 6$ in each group. A: MDA, B: GPx, C: SOD, D: TAC. * $p < 0.05$ and ** $p < 0.01$ compared to COUM 120 group. MDA: Malondialdehyde, GPx: Glutathione peroxidase, SOD: superoxide dismutase, TAC: Total antioxidant capacity, COUM: Coumestrol, Veh: Vehicle for PHTPP.

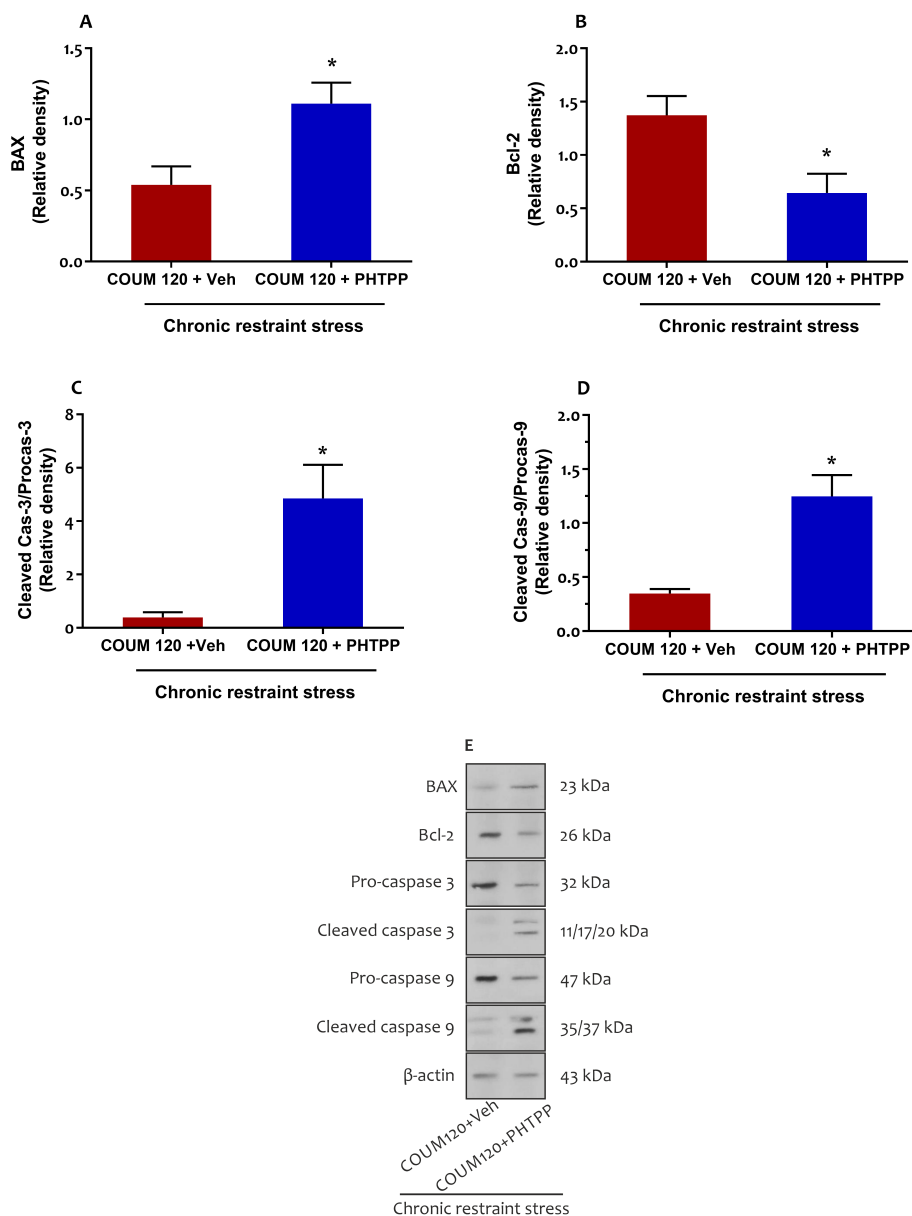


Figure 11. Effects of antagonist administration on apoptotic and anti-apoptotic parameters. $n = 3$ in each group. A: BAX, B: Bcl-2, C: Caspase-3, D: Caspase-9, E: Image of correspond blots. * $p < 0.05$ compared to COUM 120 group. BAX: Bcl-2 associated X protein, COUM: Coumestrol, Veh: Vehicle for PHTPP.

the temperature of the environment and inducing electric shocks to feet are some of the ways through which researchers simulate stressful situations for animals in their studies.¹⁸

In addition to the above, restraining has been known as one of the standard and widely used methods to induce psychological stress in rodents. It is a simple and non-invasive method which not only increases cerebral oxidative stress, inflammation and apoptosis, but also induces behavioral changes including depression, anxiety and cognitive impairments.^{1,6}

Our results showed that induction of restraint stress for 14 days would be associated with anxiety-like behaviors as indicated by reduction in center durations and the times spent in open arms (OF and EPM tests,

respectively). Moreover, HPA axis has already been found to be hyperactivated as a response to chronic stress which leads to hypersecretion of CORT in rodents. So, many negative consequences of enduring chronic stress would be mediated through dysregulations in the mentioned axis.^{1,6,46} Anxiety is a condition that can occur following exposure to stressors⁴⁷ and may lead to impairments in cognition and memory.⁴⁸ According to the results, administration of COUM reduced serum CORT levels and anxiety-like behaviors in stressed animals. In line with our data, Lund *et al.*⁴⁹ showed that administration of diarylpropionitrile (DPN) which is a well-known ER β agonist, would result in lowering plasma levels of CORT. Into the bargain, according to a study in 2017, the phytoestrogen genistein was found capable in attenuating anxiety-like behaviors

without any significant effects on locomotor activity. This compound which exerts various pharmacological benefits can also be focused regarding its potential to treat stress-related diseases in future studies.⁵⁰ Another phytoestrogen, formononetin was also found to be anxiolytic when it was evaluated in a preclinical model of pain.⁵¹ Accordingly, in present research, the lower levels of CORT in COUM-treated mice were predictable. So it was tested and as presented in the results, the effect was clearly observable.

Previous studies have elucidated that long-term exposure to stressful situations would harm different brain structures, particularly hippocampus. The damages may lead to destructive effects on memory and cognition which have already been proved and reported. For instance impairments in declarative, prospective, episodic and spatial memories can be mentioned.⁵²⁻⁵⁴ In this study, we used NOR and MWM tests which are commonly employed to evaluate episodic and spatial memories, respectively. These two aspects of memory would be impaired in the face of stressful conditions, particularly.⁵⁴ As the results indicated, it was shown that induction of chronic stress would reduce the times spent in the target quadrant (MWM test) as well as the displacement indexes (NOR test) while increasing the latency times (MWM test) significantly. Furthermore, regarding the ability of OF test in evaluating locomotor activity in one hand and the alterations in this parameter following many interventions and subsequently obtaining false results (negative or positive) on the other hand,⁵⁵⁻⁵⁸ the behavioral test gave us valuable data in this study. According to the results of the present research, locomotor activity was not statistically different between the experimental groups. Therefore, the improving effects of COUM on the studied behavioral parameters in EPM, NOR and MWM tests, would not be resulted from changes in locomotion.

As previously validated,⁶ the method was effectively able to affect episodic and spatial memories. Furthermore, based on the present data, treating the mice with COUM would improve the both. In this regard, Lee and colleagues, used the above mentioned behavioral tests for evaluating the ability of another phytoestrogen (genistein) to alleviate stress-induced cognitive impairments. Supporting our data, the aspects of memory which were defected as a result of post-traumatic stress disorder, were improved by genistein treatment significantly.⁵⁹

Expectedly, in our research chronic stress reduced GPx, SOD and TAC while increased MDA levels. These alterations warn about occurrence of oxidative stress which subsequently may lead to apoptosis.⁶⁰ The reduction in GPx, SOD and TAC as well as the elevation in MDA levels following tolerating chronic stress has been already reported.⁶ Hippocampus is one of the regions in the brain which contains a significant number of ER β s^{27,36} and can be highly affected in response to stressors.¹⁶ On the other hand, it is strongly involved in controlling cognition, learning and memory.⁶¹ Therefore, harmful effects of oxidative stress on hippocampal structure would be justifiable. Altogether

these facts suggest that the present model would be able to effectively induce oxidative stress and subsequently apoptosis leading to behavioral impairments.

Anti-apoptotic potential of COUM was also exhibited in this study as well as its anti-oxidative capacity. Analysis of oxidative stress status and anti-oxidative factors indicated the ability of COUM in attenuating the elevations in MDA and the reductions in GPx, SOD and TAC levels.

Furthermore, according to the results, in COUM-receiving groups, hippocampal levels of the anti-apoptotic factor (Bcl-2) were higher while the levels of the apoptotic factors (BAX and caspases) were lower when compared to Vehicle-treated group.

Previous studies suggested that, the anti-oxidative and anti-apoptotic effects of phytoestrogens would be mainly mediated through modulating estrogen receptors.^{62,63} Therefore, the second phase of the research was performed to investigate the possible role of ER β s in developing the beneficial effects of COUM. As a well-known selective ER β antagonist,⁶⁴ PHTPP was able to reverse almost the whole positive changes that were made in the behavioral, oxidative and apoptotic factors to a significant extent. This findings suggest the possible involvement of ER β s which play important roles in proper functioning of brain (particularly hippocampus) regarding behavioral abilities. Previous studies have suggested that brain ER β s would modulate learning and memory and do not appear to have significant effects on reproductive system.^{27,65} Studies have shown that down-regulation of estrogen receptors increases vulnerability of the neurons against destructive agents such as oxidants. Mitogen-activated protein (MAP) kinases seem to be strongly involved in the cellular pathways by which estrogen receptors protect neurons. Besides it has been reported that stimulating ER β s would increase estrogenic induction of brain-derived neurotrophic factor (BDNF)⁶⁶⁻⁶⁸ which is well-known for its neurogenic and neuroprotective properties.⁶⁹ Therefore, involvement of BDNF and MAP kinases in neuroprotective effects of COUM which is an ER β modulator seems to be very likely. As mentioned, it has been shown that, pharmacological effects of estrogens can be receptor-dependent or independent.²⁶ Evidence suggests that their anti-oxidative effects depend on the interactions with the receptors to a significant extent.⁶³ Based on these facts, involvement of ER β s in the alleviating effects of COUM (as a natural SERM) would seem very likely. The hypothesis was tested and subsequently confirmed in the second phase of the research. So ER β s can be considered as the receptors to which COUM binds and exerts its biochemical and behavioral benefits in stressful situations. Furthermore, as mentioned, MAP kinases (as well as BDNF) have been introduced to take part in the pathways through which estrogen receptors act. Therefore, their involvement in COUM mechanism of action can be a suitable target for future investigations. However, obtaining more accurate information regarding the pathways and mechanisms through which COUM acts, requires further immunohistochemistry and immunofluorescence studies.

Conclusion

According to the data obtained from this study, coumestrol may be able to improve the anxiety and the impairments in spatial and episodic memories induced by chronic stress as well as the inefficiencies in anti-oxidative and anti-apoptotic systems, through hippocampal estrogen receptor-beta. However, further immunohistochemistry and immunofluorescence studies are required to elucidate the precise cellular and molecular pathways involved.

Ethical Issues

All experiments and procedures were performed between 09:00 and 14:00, in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee for the Use of Animals in Research at Tabriz University of Medical Sciences (Code:IR.TBZMED.VCR.REC.1397.245).

Author Contributions

KF was the student who performed the whole practical procedures and wrote the manuscript. AMN and JM were the supervisors and led the project. SSE interpreted the behavioral records and FF provided constructive points and perspectives throughout the whole steps of the project. All authors discussed the results and contributed to the final manuscript and give consent for the aforementioned content to be published in this journal.

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Conflict of Interest

The authors report no conflicts of interest.

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