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EFFECT OF FLUOXETINE AND RESVERATROL ON TESTICULAR FUNCTIONS AND OXIDATIVE STRESS IN A RAT MODEL OF CHRONIC MILD STRESS-INDUCED DEPRESSION

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Our objective was to investigate the effects of chronic unpredictable mild stress (CUMS) with or without selective serotonin reuptake inhibitor (fluoxetine) and anti-oxidant (resveratrol) on testicular functions and oxidative stress in rats. Fifty male rats were divided into 2 groups; control and CUMS. CUMS group was further subdivided into 4 subgroups administered water, fluoxetine, resveratrol and both. Sucrose intake, body weight gain, serum corticosterone, serotonin and testosterone levels, sperm count and motility, testicular malondialdehyde, superoxide dismutase (SOD), catalase, glutathione (GSH), and gene expression of steroidogenic acute-regulatory (StAR) protein and cytochrome P450 side chain cleavage (P450scc) enzyme were evaluated. CUMS decreased sucrose intake, weight gain, anti-oxidants (SOD, catalase, GSH), testosterone, serotonin, StAR and cytochrome P450scc gene expression, sperm count and motility and increased malondialdehyde and corticosterone. Fluoxetine increased malondialdehyde, sucrose intake, weight gain, serotonin and decreased anti-oxidants, StAR and cytochrome P450scc gene expression, sperm count and motility, testosterone, corticosterone in stressed rats. Administration of resveratrol increased anti-oxidants, sucrose intake, weight gain, serotonin, StAR and cytochrome P450scc gene expression, testosterone, sperm count and motility, and decreased malondialdehyde and corticosterone in stressed rats with or without fluoxetine. In conclusion, CUMS induces testicular dysfunctions and oxidative stress. While treatment of CUMS rats with fluoxetine decreases the depressive behavior, it causes further worsening of testicular dysfunctions and oxidative stress. Administration of resveratrol improves testicular dysfunctions and oxidative stress that are caused by CUMS and further worsened by fluoxetine treatment.

Key words: *depression, chronic mild stress, sucrose intake, testicular functions, oxidative stress, fluoxetine, resveratrol, serotonin*

INTRODUCTION

In this modern world, stress is an unavoidable phenomenon. Involvement of stress has been suggested in the development of human depression (1). In animals, unpredictable stressors have been shown to induce changes in behavioral parameters, including changes in locomotor and explorative behavior, impairment of feeding, drinking and sexual behavior (2). Such behavioral changes are often seen in human psychiatric disorders. A regime of uncontrollable stress has been used extensively to model the deficits in motivation and reward. In DSM-IV (American Psychiatric Association 1994), anhedonia (loss of interest or pleasure in events that usually would be enjoyed) is defined as a core symptom of depression (3).

The testes carry out spermatogenesis and steroidogenesis. Leydig cells, under the stimulus of luteinizing hormone (LH), secrete testosterone (Ts) that is required by Sertoli cells to support spermatogenesis (4). Moreover, in Leydig cell steroidogenesis, the cholesterol is converted into Ts through a series of steroidogenic steps catalyzed by steroidogenic acute-regulatory (StAR) protein and cytochrome P450 side chain cleavage (P450 scc) enzyme (5). The adverse effects of stress on male reproductive system have been described. A variety of stressors such as hyperthermia and microorganisms inhibit male reproductive functions and spermatogenesis (4). Similar effects were observed after application of stressful stimuli such as prolonged immobilization (6). On the other hand, there have been relatively few studies on the effects of reactive oxygen species (ROS) on steroidogenesis. Diemer *et al.* (7) demonstrated that the in vitro exposure of MA-10 tumor Leydig cells to ROS decreased StAR protein expression levels.

Fluoxetine (FLX) is a selective serotonin reuptake inhibitor widely prescribed for treatment of neurological disorders such as depression and anxiety (8). The effect of FLX on male fertility and reproduction was studied. Long-term ingestion of FLX caused a decrease in spermatogenesis, Ts and follicle stimulating hormone (FSH) levels and weights of reproductive organs in rats (9). These sexual side effects can considerably affect a person's lifestyle, and where this results in reduced compliance with medication, lead to less effective treatment of the primary psychiatric disorder (10).

Resveratrol (RES) is a phytoalexin with antioxidant properties and is found in a wide range of foods especially grapes and red wine. During the last decade,resveratrol has been shown to possess wide spectrum of pharmacologic properties such as anti-inflammatory, antioxidant, anticarcinogenic, antiageing, neuroprotective and cardioprotective effect (11). *In vivo* and *in vitro* studies have shown that RES protects spermatocytes against lipid peroxidation and increases testicular sperm numbers and sperm motility (12). Also, it increases sperm production, reduce apoptosis in germinal cells, and protect against environmental toxins (12). However, few studies deal with the relationship between RES and the possible decrease in testicular functions provoked by chronic unpredictable mild stress (CUMS)-induced depression, with or without FLX treatment.

Therefore, the aim of the current work was to determine the effects of CUMS-induced depression with or without FLX on testicular functions and oxidative stress in adult male rats as well as the gene expression of testicular steroidogenic enzymes. Also, we investigated whether RES could protect the testes against the possible negative effects of CUMS or FLX.

MATERIALS AND METHODS

Chemicals

FLX (Philozac®) was obtained as capsules from Amoun Pharmaceutical Company, Cairo, Egypt. Each capsule contained 10 mg FLX hydrochloride. FLX was dissolved in distilled water and was given orally to animals by gavage at a dose level of 10 mg/kg body weight, (equivalent to the therapeutic dose for human according to Paget and Barnes (13)), daily for four weeks. RES was purchased from Sigma (St. Louis, Mo., USA) and given by gavage in a dose of 20 mg/kg body weight daily for four weeks.

Animals

Fifty male Sprague Dawley rats, 7 weeks of age, weighing 180 – 220 g were purchased from the Vaccine and Immunization Authority (Helwan, Cairo, Egypt) and housed (Animal House, Medical Physiology Department, Faculty of Medicine, Mansoura University, Egypt) under controlled conditions (temperature of 23 ± 1°C, and a 12 h light: 12 h dark cycle). The animals were allowed free access to food and tap water. The first experimental phase ("habituation") lasted for 7 days during which body weight was recorded daily.

Experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). All experimental procedures in this study were approved by the Medical Research Ethics Committee of Mansoura University, Egypt.

Experimental design

Animals were randomly divided into the control (n = 10) and stress (n = 40) groups. Control rats received distilled water orally for 28 days. The stress group, subjected to CUMS procedure, was further subdivided into 4 subgroups, ten animals each. The first subgroup received distilled water orally for 28 days. The other subgroups were treated with anti-depressant FLX (10 mg/kg/day for 28 days, through gavage) (14), RES (20 mg/kg through gavage) (15) or both FLX (10 mg/kg/day, through gavage) + RES (20 mg/kg through gavage) for 28 days. The administration of drugs was initiated concurrently with CUMS procedure.

Stress procedure

A rat model of CUMS-induced depression was established from the procedure described by (3, 16, 17). After the seven days habituation period, all rats (except the normal) were subjected to a series of chronic unpredictable stimuli for four weeks. Each week consisted of tail clamping for 1 min, cold water swimming at 4°C for 5 min, heat stress at 45°C for 5 min, paired caging for 2 hours, tilted cage (45°) for 3 hours, food deprivation (18 h) immediately followed by 1 hour of restricted access to food (five micropellets), water deprivation (18 h) immediately followed by 1 hour exposure to an empty bottle, wet cage (200 ml water in 100 g sawdust bedding) for 21 hours, and 24 hours of reversed light/dark cycle (7:00 a.m. lights off, 7:00 p.m. lights on). All animals received the same pattern of stressors. All stressors were applied individually and continuously day and night. Their sequence was at random in order to be completely unpredictable to the animal. Immediately following each stress session the animals were returned to their home cages and maintained under standard conditions until the next stress session. Control animals were left undisturbed in their room and home cages. They were deprived of food and water for 4 hours preceding each sucrose test, but otherwise food and water were freely available in the home cage.

Sucrose intake and body weight

In the present study, we used a sucrose test to assess anhedonia. Sucrose intake (1% sucrose solution) was measured once a week, on separate days (on days 0, 7, 14, 21, and 28), during a 1-h window after 4 h of food and water deprivation. Consumption was measured by comparing bottle weight before and after the 1-h window. Baseline was measured 1 week before the start of chronic stress. The food and water deprivation period preceding sucrose intake measurement may be considered as a further stress applied on top of the chronic stress protocol. However, control rats were also exposed to the food and water deprivation, as a part of the test (3). The body weights were recorded regularly throughout the experiments.

Forced swimming test (FST)

At the end of the experiment, the depressive behavior induced by forced swimming test was evaluated in all animals using the modified method of Porsolt *et al.* (18). This procedure consists of exposing an animal to a situation of inescapable stress, in which the rat is forced to swim. After an initial period of vigorous swimming activity in the direction to the tank border (Latency of the Attempt of Escape), the animal reduces the intensity of the movements, just producing the necessary movements to maintain its head out of the water. This answer was classified as behavioral immobility, indicating a possible state of despair of the animal when it realizes that there is no escape.

The rats were placed individually in a cylindrical tank (100 cm diameter × 60 cm height) whose level of water do not allow the animal to lean on the floor, nor arise by the border. The temperature of the water was maintained in 25°C. The animals were submitted to the forced swimming during 15 min (pre-test). After the 15 min of forced swimming, each animal was gently dried and then returned to their cages. Twenty four hours after the pre-test, all the animals were put back inside of the tank. The individual behavioral evaluation was accomplished and quantified during 5 min of forced swimming. The behavioral parameters as latency of the attempt of escape (LAE) and behavioral immobility (BI) were quantified in seconds (s). Then the animals were remove from the water and gently dried and placed back into their cages.

Sampling Protocol

1. Blood samples

At the end of the experiment, all animals were sacrificed by decapitation under ether anesthesia and blood samples were collected without anticoagulant, left for 10 min and then centrifuged for 10 min at 4000 r/min to obtain serum, which was used for determination of Ts and corticosterone concentrations.

2. Tissue samples

Testicular tissue preparation: Testicular tissues were removed, rinsed with cold normal saline, divided into parts and dried with filter paper. First part was quickly frozen in liquid nitrogen then stored at –80°C for determination of oxidative stress parameters and semiquantitative reverse transciptase (RT)-PCR of steroidogenic enzymes. The other part was used for histopathological evaluation. In addition, the epididymis was processed for assessment of the sperm parameters.

3. Brain tissue preparation

The whole brain was quickly removed and washed in 0.9% cold saline. The cortex and hippocampus were carefully dissected and homogenized. The homogenates were stored at –80°C until used for determination of serotonin level.

4. Sperm count and motility

To determine these parameters, cauda epididymis was minced with a scalpel blade in a Petri dish into 5 ml of 0.9% NaCl pre-warmed to 37°C. One drop of evenly mixed sample was applied to a specific slide, and the motility/concentration module of the computer assisted semen analysis CASA system using MiraLab - Egypt (Mira 9000 sperm Analyzer CASA software). For analysis, trinoculer microscope with plan objective lens (olympus) equipped with phase contrast optics and a heated stage (37°C) was used. Sperm count was expressed as million per ml. The number of motile spermatozoa was expressed as percentage of sperm motility. In addition, morphology was assessed by the preparation of a smear and the application of the sperm Mac stain staining method (Fertipro, Bilguim) as outlined by WHO (2010). The sperms were classified into normal and abnormal and the total sperm abnormality was expressed as percentage incidence.

Biochemical investigations

ELISA assay kits were used for determination of serum Ts (Cat. No: 55-TESMS-E01, ALPCO Diagnostics, USA) and corticosterone (Cat No: 55-CORMS-E01, ALPCO Diagnostics, USA) levels according to the manufacturer's instructions. In addition, the serotonin level in the brain tissue (cerebral cortex and hippocampus) homogenate was assayed using ELISA kit (Catalog No: MBS725497, MyBioSource, USA) according to the manufacturer's instructions.

Estimation of testicular oxidative and anti-oxidative parameters

Parts of the frozen testicular samples from all groups were washed with phosphate buffered saline (PBS), pH 7.4, containing 0.16 mg/ml heparin to remove any red blood cells and clots. Then, they were homogenized in cold phosphate buffer, pH 7.0, containing EDTA Homogenates were centrifuged at 10,000 g for 15 min at 4°C, and the supernatant was kept at –80°C until used for analysis of lipid peroxidation product (MDA), catalase, superoxide dismutase (SOD) and glutathione (GSH), which were measured by using colorimetric kits (Bio-Diagnostics, Dokki, Giza, Egypt) according to the manufacturer's instructions.

Semiquantitative reverse transcriptase-PCR of steroidogenie enzymes

Total RNA extraction was carried out from rat testis, after homogenization, using TriFast TM reagent (PeqLab. Biotechnologie GmbH, Carl-Thiersch St. 2B 91052 Erlongen, Germany, Cat. No. 30-2010) according to the manufacturer's instructions. The remaining DNA was removed by digestion with DNase I (Sigma). The RNA purity and concentration were determined spectrophotometrically at A260/A280 nm. The purity of RNA we obtained was 1.8 – 1.9.

Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using ready-to-go RT-PCR beads for first cDNA synthesis and PCR reaction provided by Amersham Biosciences, England. Cat. No. 27-9266-01, according to the method of Berchtold (19).

Gene specific primers were purchased from Biolegio. BV, PO Box 91, 5600 AB Nijmegen, Netherlands. The primers used for RT-PCR for StAR protein and cytochrome P450scc were gene-specific primers selected according to Akingbemi *et al.* (20).

StAR protein: forward primer; 5'-TTG GGC ATA CTC AAC AAC CA-3'; reverse primer; 5'-ATG ACA CCG CTT TGC TCA G-3'.

Cytochrome P450scc: forward primer; 5'-AGG TGT AGC TCA GGA CTT-3'; reverse primer; 5'-AGG AGG CTA TAA AGG ACA CC-3'.

RPS 16 (internal control, house keeping gene): forward primer; 5'-AAG TCT TCG GAC GCA AGA AA-3'; reverse primer ; 5'-TTG CCC AGA AGC AGA ACA G-3'.

Thermal cycling reaction was performed using thermal cycler (TECHEN TC-312, Model FTC3102D, Barloworld Scientific Ltd. Stone, Stafford Shire, st 150 SA, UK) with the following program: initial denaturation: 94°C for 5 min, 35 cycles of 94°C for 0.5 min denaturation, 60°C for 1 min (StAR protein), 58°C for 30 s (cytochrome P450scc), and 60°C for 1 min (RPS 16) as primer annealing, 72°C for 1 min extension and final extension at 72°C for 7 min. The products was subjected to agarose gel electrophoresis using 2% agarose stained with ethidium bromide and visualized *via* light UV transilluminator (Model TUV-20, OWI Scientific, Inc. 800 242-5560, France) and photographed under fixed conditions (the distance, the light and the zoom). The RT-PCR products size were as follow: 389, 399 and 148 bp for StAR protein, cytochrome P450scc and RPS 16 (internal control-house keeping gene) respectively. The results photos were analyzed with Scion Image® release Alpha 4.0.3.2. Software for Windows® which performs bands detection and conversion to peaks. Area under each peak were calculated in square pixels and used for quantification. Gene expression levels were determined by calculating the ratio between the square pixel value of the target gene in relation to the control gene (house keeping gene).

Histopathological examination of the testes

Specimens from testes were fixed in 10% v/v neutral buffered formalin, dehydrated through ascending grades of ethanol (70, 90 and 95% v/v), cleaned in xylene and embedded in paraffin wax (melting point 56°C). Five µm sections were stained with hematoxylin and eosin (H-E) before investigation under light microscopy. Histological findings in seminiferous tubules were evaluated according to Johnsen's scoring system (21). Tubuli in 10 consecutive 400 × field areas were scored and mean values were determined. The Johnsen score is based on the premise that with

testicular damage there is successive disappearance of the most mature cell type, with progressive degeneration of germinal epithelium, with the disappearance of spermatozoa and spermatids, then spermatocytes and finally Sertoli cells, in that order.

A score of 1 to 10 was given to each tubule according to the maturity of the germ cells: a score of 1 indicated no seminiferous epithelial cells and tubular sclerosis. A score of 2 indicated no germ cells, only Sertoli cells. A score of 3 indicated spermatogonia only. A score of 4 indicated no spermatids, few spermatocytes, and arrest of spermatogenesis at the primary spermatocyte stage. A score of 5 indicated no spermatids and many spermatocytes. A score of 6 indicated no late spermatids, few early spermatids, arrest of spermatogenesis at the spermatid stage, and disturbance of spermatid differentiation. A score of 7 indicated no late spermatids and many early spermatids. A score of 8 indicated few late spermatids. A score of 9 indicated many late spermatids and disorganized tubular epithelium. A score of 10 indicated full spermatogenesis.

Statistical analysis

The data were expressed as mean ± standard deviation (S.D.). Data were processed and analyzed using the SPSS version 10.0 (SPSS, Inc., Chicago, Ill., USA). For analysis of sucrose intake, two-way ANOVA was done followed by the Tukey's multiple comparison test. For analysis of weight gain, corticosterone, Ts and serotonin levels as well as oxidative and anti-oxidative parameters, gene expression and Johnsen's score in rat testis, one-way ANOVA was done followed by Tukey's post hoc test. Pearson correlation statistical analysis was done for detection of a probable significance between two different parameters. Results were considered significant if $P \leq 0.05$.

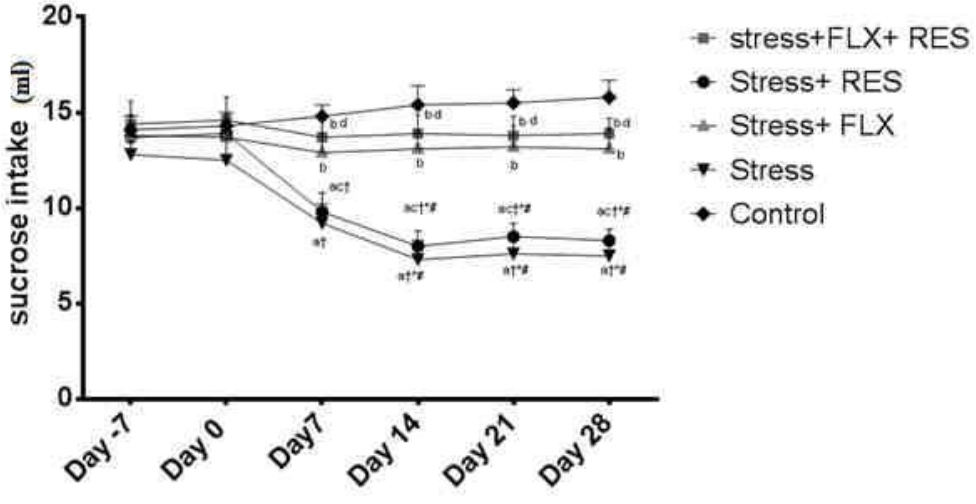
RESULTS

Sucrose intake in control, CUMS rats and CUMS rats administered FLX, RES and combination of both FLX and RES are shown in *Fig. 1*. Before CUMS, there was no significant difference in sucrose intake among the different groups ($P > 0.05$). CUMS induced a significant decrease in sucrose intake in the stress group as compared with the normal control group ($P < 0.05$). While RES had no significant effect on sucrose consumption in CUMS rats, administration of FLX to CUMS or CUMS +RES groups significantly increased sucrose consumption ($P < 0.05$) in comparison with untreated or RES treated CUMS rats (*Fig. 1*). Tests of main effects for the period of CUMS procedure (day – 7 to day 28) showed statistically significant differences among the five groups of animals (control, CUMS, and CUMS with FLX, RES and both) ($F_{(4.273)} = 376.3$, $P < 0.0001$). There was a significant interaction between group and day ($F_{(20.273)} = 25.02$, $P < 0.0001$).

Body weight gain in control, CUMS rats and CUMS rats administered FLX, RES and combination of both are shown in *Fig. 2*. While the body weight gain, at second, third and forth weeks, was significantly ($P < 0.05$) decreased in rats subjected to CUMS with or without RES when compared with control group, FLX administration either alone or with RES significantly ($P < 0.05$) increased weight gain in CUMS exposed rats as compared with untreated or RES treated stressed rats. The weight gain in FLX treated stressed rats showed normal values similar to those of the control normal group ($P > 0.05$).

The behavioral parameters during the FST as LAE and BI were appraised (*Fig. 3*). LAE of the stress group was significantly smaller ($P < 0.05$) while BI was larger ($P < 0.05$) when compared with the control group. While RES had no significant effect on LAE and BI in CUMS rats, administration of FLX to CUMS or CUMS + RES groups significantly increased LAE and decreased BI ($P < 0.05$) in comparison with untreated or RES treated CUMS animals (*Fig. 3*).

Table 1 show the levels of corticosterone and Ts in serum, serotonin in cerebral cortex and hippocampus as well as sperm count, motility and abnormality in control, CUMS and CUMS with FLX, RES and combined FLX and RES groups. A significant decrease in the serum Ts level was observed in CUMS group as compared with the control group ($P < 0.05$). FLX administration to CUMS rats caused a further decrease in Ts level as compared with untreated CUMS group ($P < 0.05$). Moreover, RES treatment either alone or with FLX significantly increased serum Ts level (P



ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	459.2	20	22.96	F (20, 273) = 25.02	P < 0.0001
Row Factor	280.6	5	56.11	F (5, 273) = 61.16	P < 0.0001
Column Factor	1381	4	345.3	F (4, 273) = 376.3	P < 0.0001

Fig. 1. Sucrose intake (ml) during the period of chronic stress in rats.
Data are expressed as mean ± S.D. [a] significant ($P < 0.05$) versus control; [b] significant ($P < 0.05$) versus stress group; [c] significant ($P < 0.05$) versus stress + FLX group; [d] significant ($P < 0.05$) versus stress + RES group; [†] significant ($P < 0.05$) versus their baseline (day 7); * significant ($P < 0.05$) versus day 0; [#] significant ($P < 0.05$) versus day 7.

< 0.05) in CUMS rats as compared with untreated or FLX treated stressed rats (*Table 1*). Also, in response to repeated stress, serum corticosterone increased while serotonin level in cortex and hippocampus decreased significantly (P < 0.05) as compared with the control group. On the other hand, in FLX or combined FLX + RES treated CUMS rats, the corticosterone decreased while serotonin increased (P < 0.05) significantly when compared with the untreated stress group (*Table 1*).

While both sperm concentration (million/ml) and progressive sperm motility (%) showed significant decrease (P < 0.05) in CUMS group when compared to the control group, sperm abnormality was significantly (P < 0.05) increased. FLX caused a further decrease in sperm concentration and progressive sperm motility and increase in abnormal sperms in CUMS rats when compared with untreated stress group (P < 0.05). In addition, treatment with RES significantly increased sperm concentration and motility and decreased abnormality in CUMS and CUMS + FLX groups when compared with untreated or FLX treated stressed rats (*Table 1*).

The changes in MDA and GSH levels and catalase and SOD activities are presented in *Table 2*. While antioxidant enzyme activity (SOD and CAT) and GSH were significantly decreased in CUMS group as compared with control group, MDA significantly increased (P < 0.05). Administration of FLX to CUMS rats caused a further increase in MDA and decrease in antioxidant enzyme activity (SOD and CAT) and GSH as compared to the untreated stressed animals (P < 0.05). Moreover, RES significantly increased antioxidant enzyme activity (SOD and CAT) and GSH level and decreased MDA in CUMS and CUMS + FLX groups (P < 0.05) when compared with the untreated or FLX treated stressed rats (*Table 2*). In addition, serum Ts level showed statistically significant negative correlation with testicular MDA levels (r = –0.8757; P < 0.001) (n = 50) (*Fig. 4*).

Fig. 5 showed that testicular gene expression of StAR and cytochrome P450scc was significantly decreased (P < 0.05) in CUMS group when compared to the control group. FLX administration caused a further decrease in testicular gene expression of StAR and cytochrome P450scc in CUMS rats as

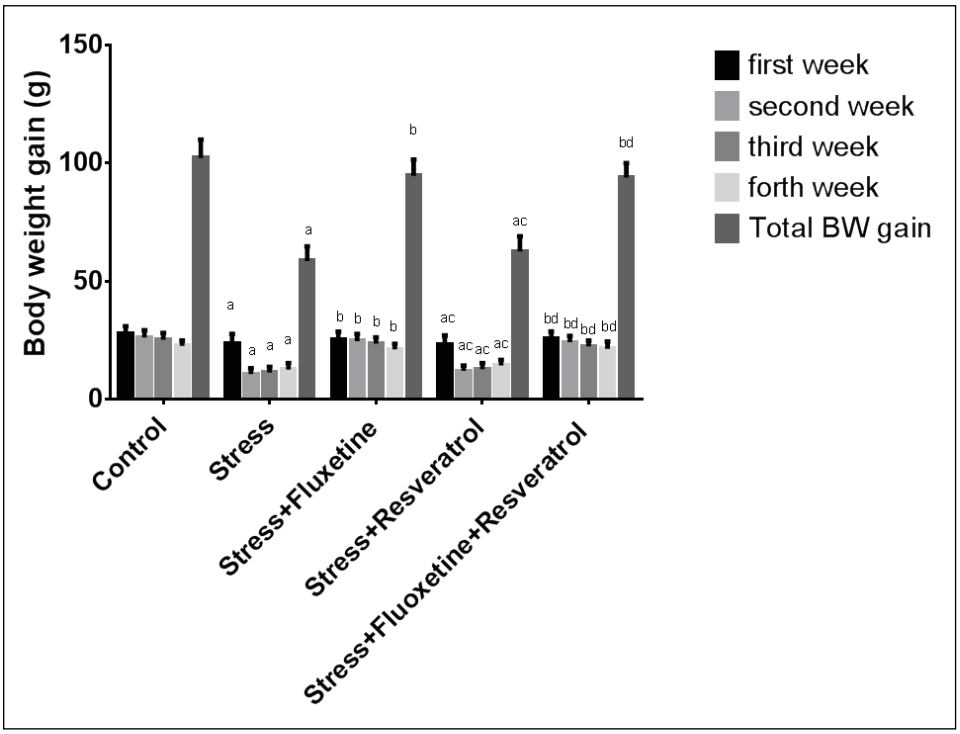


Fig. 2. Body weight gain (g) of control, stress, and stress with FLX, RES or combined FLX and RES groups.
Data are expressed as mean ± S.D. FLX, fluoxetine; RES, resveratrol; [a] significant (P < 0.05) versus control; [b] significant (P < 0.05) versus stress group; [c] significant (P < 0.05) versus stress + FLX group; [d] significant (P < 0.05) versus stress + RES group.

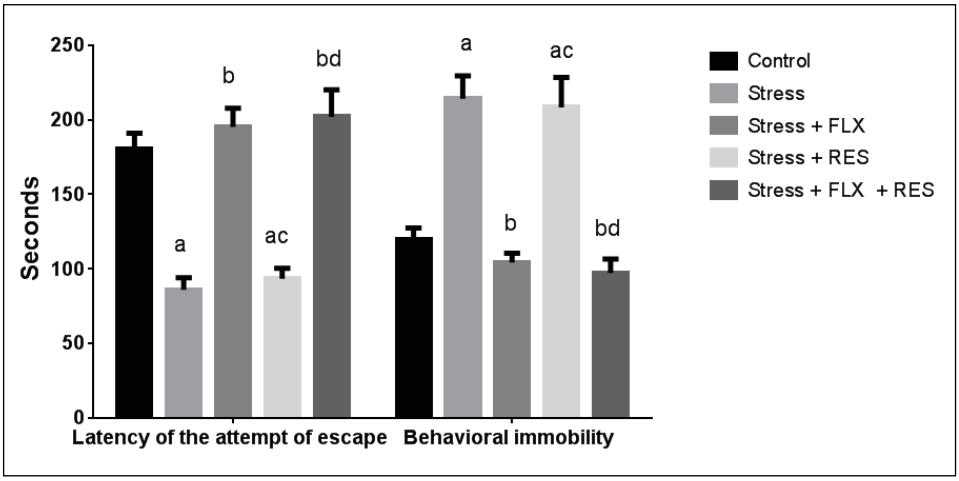


Fig. 3. Latency of the attempt of escape (LAE) and behavioral immobility (BI) in rats of the control, stress, and stress with FLX, RES or combined FLX and RES groups.
Data are expressed as mean ± S.D. FLX, fluoxetine; RES, resveratrol; [a] significant (P < 0.05) versus control; [b] significant (P < 0.05) versus stress group; [c] significant (P < 0.05) versus stress + FLX group; [d] significant (P < 0.05) versus stress + RES group.

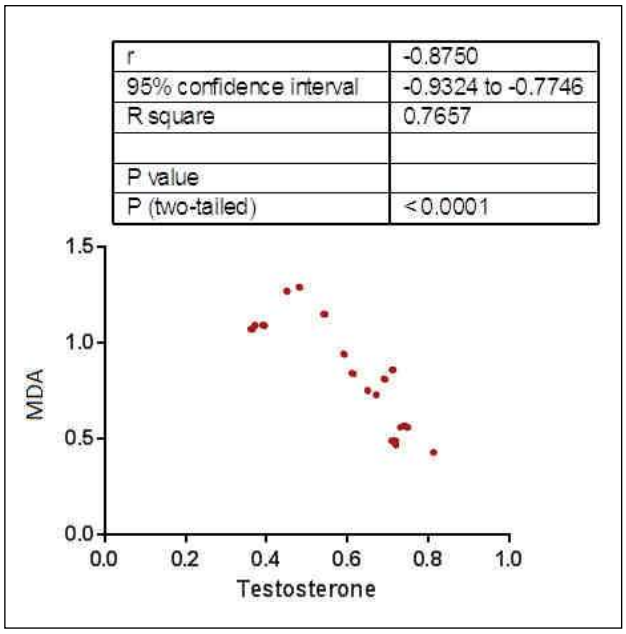


Fig. 4. Pearson correlation coefficient between serum testosterone level and testicular MDA (r = –0.8750) (P < 0.001) (n = 50).

compared with untreated stress group (P < 0.05). In addition, a significant increase in the testicular gene expression of StAR and cytochrome P450scc was detected in RES treated groups (CUMS + RES and CUMS + FLX + RES) when compared with stress groups untreated or treated with FLX (*Fig. 5*).

Histopathological findings

The seminiferous tubules of the control group were completely differentiated. Sections of testis from the control group revealed that seminiferous tubule had each a definite membrane and a small lumen densely filled with sperm tails. Spermatogenic cells including spermatogonia, primary spermatocytes, early spermatids, late spermatids and Sertoli cells were seen to be abundant and healthy (*Fig. 6A*). On the other hand, examination of the testes of CUMS rats, revealed degeneration of spermatogonial cells lining the seminiferous tubules and that the lumens of tubules were filled with degenerated germ cells. Also, vacuolization of the seminiferous epithelium and partial to complete absence of germ cells associated with interstitial edema, damaged Sertoli cells and necrosis of Leydig cells were also noticed (*Fig. 6B*). The testes of CUMS rats treated with FLX (*Fig. 6C*) revealed histopathological changes similar to those observed in untreated CUMS group. However, morphological examination of the testes in RES treated CUMS groups, either with or without FLX (*Fig. 6D* and *6E*), revealed similar morphology to that of the control group. The

Table 1. Serum corticosterone and testosterone, serotonin level in cerebral cortex and hippocampus and sperm parameters in control, stress and stress with FLX, RES or combined FLX and RES groups.

	Control (n = 10)	Stress (n = 10)	Stress + FLX (n = 10)	Stress + RES (n = 10)	Stress + FLX + RES (n = 10)
Serum corticosterone (ng/ml)	165.7 ± 13.4	348.6 ± 20.4^{a}	245.7 ± 18.8^{ab}	338.6 ± 21.5^{ac}	237.7 ± 18.8^{abd}
Cerebral cortex serotonin (ng/ml)	0.089 ± 0.007	0.041 ± 0.006^{a}	0.067 ± 0.008^{ab}	0.045 ± 0.009^{ac}	0.071 ± 0.006^{abd}
Hippocampus serotonin (ng/ml)	0.198 ± 0.008	0.085 ± 0.007^{a}	0.138 ± 0.009^{ab}	0.089 ± 0.006^{ac}	0.145 ± 0.008^{abd}
Serum testosterone (ng/ml)	1.9 ± 0.1	0.6 ± 0.1^{a}	0.3 ± 0.1^{ab}	1.4±0.1^{abc}	1.2 ± 0.1^{abc}
Sperm count (million/ml)	20.8 ± 1.5	8.1 ± 0.9^{a}	4.8 ± 0.7^{ab}	12.7± 1.2^{abc}	14.3 ± 1.3^{abc}
Sperm motility (%)	75.6 ± 3.1	34.5 ± 2.5^{a}	22.1 ± 1.8^{ab}	49.5 ± 2.7^{abc}	52.6 ± 2.6^{abc}
Sperm abnormality (%)	2.31 ± 0.05	15.64 ± 0.51^{a}	19.53 ± 0.63^{ab}	9.43 ± 0.47^{abc}	8.6 ± 0.42^{abc}

Data are expressed as mean ± S.D. FLX, fluoxetine; RES, resveratrol; ^{a} P < 0.05 versus control; ^{b} P < 0.05 versus stress group; ^{c} P < 0.05 versus stress + FLX group; ^{d} P < 0.05 versus stress + RES group.

Table 2. Testicular SOD and CAT activities, GSH and MDA levels in control, stress and stress with FLX, RES or combined FLX and RES groups.

	Control (n = 10)	Stress (n = 10)	Stress + FLX (n = 10)	Stress + RES (n = 10)	Stress + FLX + RES (n = 10)
MDA (nmol/g tissue)	4.2 ± 0.3	9.5 ± 0.5^{a}	13.7 ± 0.^{ab}	4.9 ± 0.6^{bc}	5.2 ± 0.9^{bc}
SOD (U/g tissue)	5.51 ± 0.32	3.42 ± 0.27^{a}	2.88 ± 0.15^{ab}	4.89 ± 0.35^{bc}	4.79 ± 0.37^{bc}
CAT (U/g tissue)	5.91 ± 0.31	3.72 ± 0.28^{a}	2.91 ± 0.31^{ab}	5.32 ± 0.25^{bc}	5.21 ± 0.19^{bc}
GSH (mmol/L)	48.28 ± 5.74	26.82 ± 2.41^{a}	18.14 ± 2.11^{ab}	44.16 ± 3.15^{bc}	41.35 ± 3.01^{bc}

Data are expressed as mean ± S.D. MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione; FLX, fluoxetine; RES, resveratrol; ^{a} P < 0.05 versus control; ^{b} P < 0.05 versus stress group; ^{c} P < 0.05 versus stress + FLX group; ^{d} P < 0.05 versus stress + RES group.

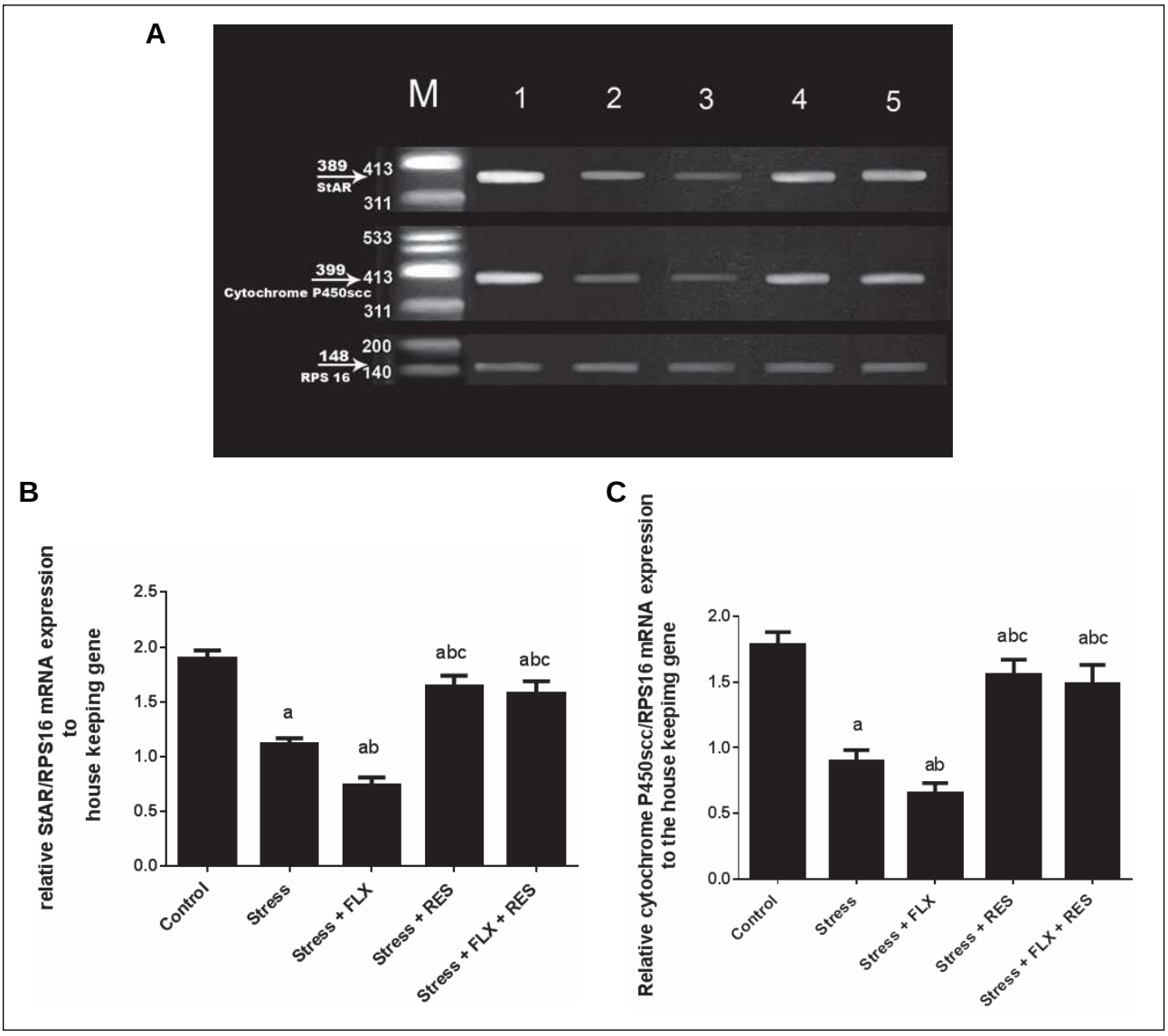


Fig. 5. (*A*) RT-PCR product of testicular StAR, cytochrome P450scc and RPS16 expression in rats of all groups. Lane M: the DNA marker; Lane 1: RT-PCR products in control group (I); Lane 2: RT-PCR products in stress group; Lane 3: RT-PCR product in stress + fluoxetine group; Lane 4: RT-PCR product in stress + resveratrol group and Lane 5: RT-PCR product in stress + fluoxetine + resveratrol group. StAR: steroidogenic acute-regulatory protein; cytochrome P450scc: cytochrome P450 side chain cleavage enzyme.
(*B*): Testicular expression of StAR/RPS16 mRNA in control, stress and stress with FLX, RES or combined FLX and RES groups. Data are expressed as mean ± S.D. FLX, fluoxetine; RES, resveratrol; [a] significant ($P < 0.05$) versus control; [b] significant ($P < 0.05$) versus stress group; [c] significant ($P < 0.05$) versus stress + FLX group; [d] significant ($P < 0.05$) versus stress + RES group.
(*C*) Testicular expression of cytochrome P450scc/RPS16 mRNA in control, stress and stress with FLX, RES or combined FLX and RES groups. Data are expressed as mean ± S.D. FLX, fluoxetine; RES, resveratrol; [a] significant ($P < 0.05$) versus control; [b] significant ($P < 0.05$) versus stress group; [c] significant ($P < 0.05$) versus stress + FLX group; [d] significant ($P < 0.05$) versus stress + RES group.

testes in RES administered groups showed nearly normal testicular morphology and spermatogenesis with almost normal seminiferous tubule architectures, disappearance of vacuolation in all stages of spermatogenesis and well-preserved Sertoli cells. In addition, spermatogonia, primary spermatocytes, spermatids, and mature sperm were clearly seen in the seminiferous tubules of RES treated rats, with increased dense packing of mature sperm in the lumen and a decreased number of sperm heads. Also, decreased interstitial edema was observed in these RES administered groups (*Fig. 6D* and *6E*).

On the other hand, Johnsen's score (*Table 3*) showed a significant decrease in CUMS groups in comparison with control rats ($P < 0.05$). FLX administration to CUMS rats caused a further decrease in the score ($P < 0.05$) as compared to untreated stress group. Treatment with RES significantly increased the score in CUMS and CUMS + FLX groups when compared to the untreated or FLX treated CUMS groups ($P < 0.05$).

DISCUSSION

Increasing evidence suggests that stress plays an important role in the development of depression. The cellular and molecular mechanisms of stress-induced depression are not completely understood (22). Stress induced adrenal corticosteroid release, which, in turn, impaired the cortical function (e.g., hippocampus), leading to depression (23). Also, a decrease in the synaptic levels of serotonin (5-hydroxy

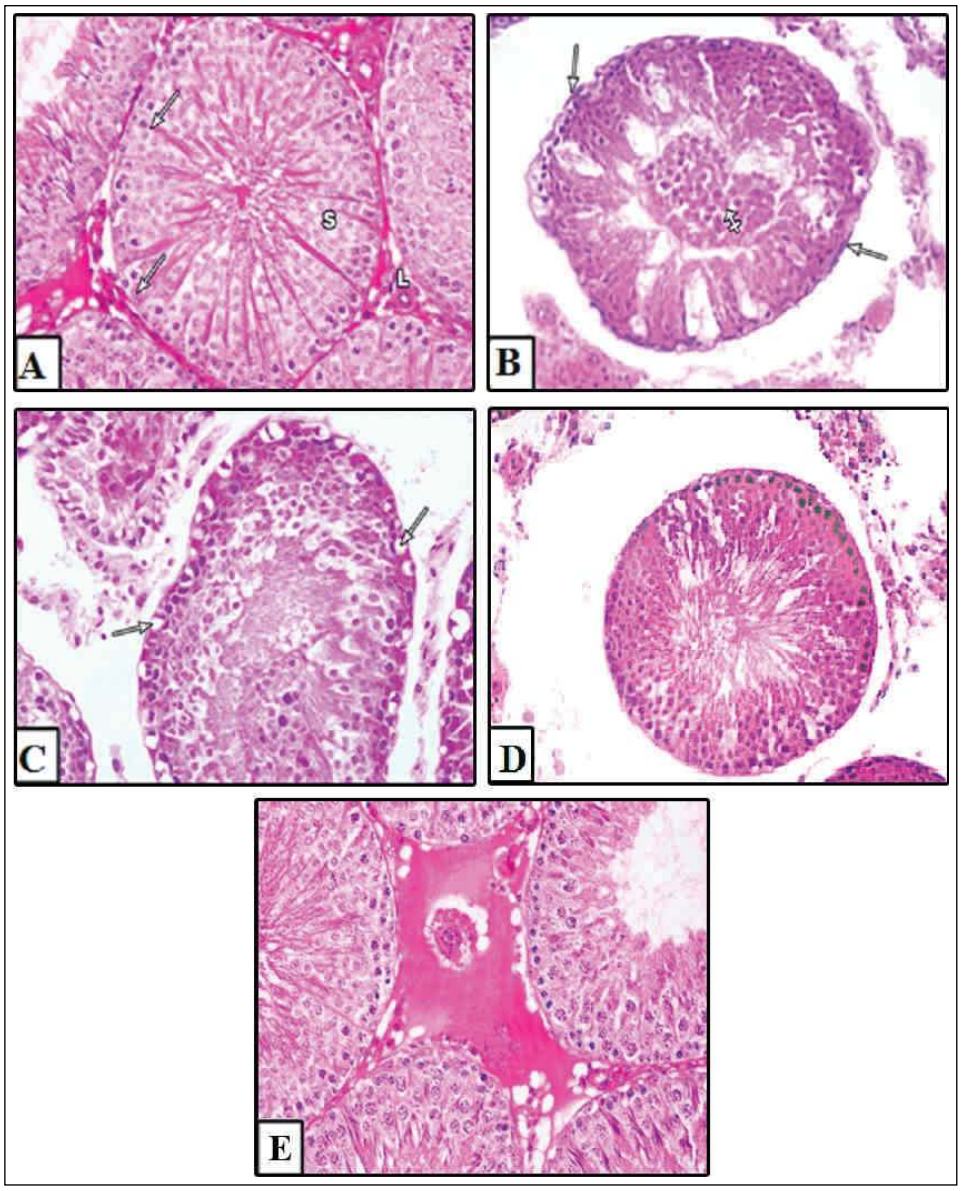


Fig. 6. Histological appearance of testis sections obtained from all groups (*A*): control rat, (*B*): stress rats, (*C*): stress with fluoxetine, (*D*): stress with resveratrol and (E): stress with fluoxetine and resveratrol; × 200.

Table 3. Johnsen's score in control, stress and stress with FLX, RES or combined FLX and RES groups.

	Control (n = 10)	Stress (n = 10)	Stress + FLX (n = 10)	Stress + ES (n = 10)	Stress + FLX + RES (n = 10)
Johnsen's score	9.7 ± 0.2	6.8 ± 0.1 [a]	6.1 ± 0.1 [ab]	8.8 ± 0.1 [abc]	8.5 ± 0.1 [abc]

Data are expressed as mean ± S.D. FLX, fluoxetine; RES, resveratrol; [a] $P < 0.05$ versus control; [b] $P < 0.05$ versus stress group; [c] $P < 0.05$ versus stress + FLX group; [d] $P < 0.05$ versus stress + RES group.

tryptamine, 5 HT) and/or norepinephrine in various brain regions, e.g. frontal cortex, as well as a reduced production of brain-derived neurotrophic factor in the hippocampus may also contribute to the stress-induced depression (24). In addition, stress can activate an inflammatory response in the brain which could induce neurochemical changes and behavioral disturbances causing a diagnosis of depression (25).

Depression is often associated with physiological changes characteristic of a normal stress response. There has been a major focus on the role of the hypothalamo-pituitary-adrenal (HPA) axis as a marker of the stress response. Stress response activates the HPA axis and the release of glucocorticoids, which increases the heart rate, blood pressure, and metabolism. A consistent finding in depressed patients is hyperactivity and dysregulation of the HPA-axis, demonstrated by increased cortisol levels, enlargement of the pituitary and adrenal glands and decreased glucocorticoid receptor sensitivity (26). Previous studies have shown that the inhibition of HPA axis by circulating glucocorticoids is impaired both in CMS rats (27) and in depressed patients (28). Moreover, considering the inhibitory role of the hippocampus in glucocorticoid synthesis, stress-induced hippocampal atrophy may have contributed to high corticosterone levels following prolonged immobilization stress (6). In agreement with these studies, the results of the current work showed a significant increase in plasma corticosterone level in CUMS-exposed rats (*Table 1*) which confirm the state of stress. Antidepressants reverse a number of the HPA-axis abnormalities.

The chronic FLX treatment normalizes the corticosterone secretion in depressed patients or experimental models. In accord with these data, our results demonstrated a significant decrease in plasma corticosterone level in CUMS-exposed rats with FLX administration either alone or with RES (*Table 1*).

In our research, we used CUMS induced depression in rats as a realistic analogue of the strains of everyday life. After 28 days of CUMS, the rats showed behavioral deficits including reduced sucrose consumption and decreased weight gain (*Figs. 1* and *2*, respectively), all of which mimic the symptoms of depression in human and indicated success in depressing the rats. These findings support CUMS as an animal model of depression. During the same period, the FLX rats showed the reverse of almost all the behavior alterations observed in stress group (*Figs. 1* and *2* respectively).

Our protocol for the consumption of sweet solutions included 4 hours of water deprivation. The choice of a 4 h limit for the water deprivation period was motivated by the fact that dehydration produces metabolic effects unrelated to the CMS protocol (3). In the CUMS-exposed rats we observed a significant decrease in the level of sucrose intake as compared with control group with maximum reduction seen after 2 weeks and lasted throughout the 4 weeks of CUMS protocol (*Fig. 1*). On the other hand, sucrose intake in the control group did not change. The current results are consistent with previous studies (3, 29). The sucrose intake lowering effect of CUMS in rats was reversed by the administration of FLX either alone or with RES (*Fig. 1*). However, RES alone had no effect on sucrose intake in stressed rats (*Fig. 1*). The results of this study confirm earlier reports (3, 30). Moreover, CUMS significantly decreased the body weight gain of rats as compared with control group. FLX treatment either alone or with RES significantly increased the body weight gain of CUMS-exposed rats (*Fig. 2*). These results were in accord with those of previous studies (12, 31). It has been suggested by some investigators that reduction of body weight following CMS contributes to a decrease in sucrose intake (32). However, in our study, we did not find any correlation between body weight and sucrose intake.

The 5-HT is involved in the neurobiology of depression, as well as in the action mechanisms of antidepressant agents. The decrease in the concentrations of brain serotonin can precipitate the recurrence of the depression in depressed patients (33). Consistent with these findings, our results showed a significant decrease in brain serotonin level in CUMS-exposed rats when compared with control group (*Table 1*) which confirm the depression in those rats. The antidepressant actions of FLX are thought to be mediated by the inhibition of serotonin reuptake, resulting in enhanced serotonergic signaling and up-regulation of brain-derived neurotrophic factor gene expression (34). In agreement with these data, the results of the current study showed a significant increase in brain serotonin in CUMS exposed rats after FLX administration either alone or with RES (*Table 1*). However RES alone caused no change in brain serotonin in stressed rats (*Table 1*). The FST has been used to evaluate the effectiveness of several antidepressant treatments (35). In a study by Page *et al.* (35), they reported that the reduction of depressant behavior in the forced swimming test in the FLX treated rats seems to be related to the function of the serotonergic system. Moreover, in another study, Mendes-da-Silva *et al.* (36) demonstrated that the decrease in depressant behavior in FST evaluated in adult life after neonatal treatment with FLX may be due to neuroadaptive mechanisms developed at the time of treatment, that persist until adult life. In accord with these data, the results of the current work showed a significant decrease in LAE and increase in BI in CUMS rats (*Fig. 3*) suggesting the development of a depression-like behavior. In addition, FLX administration to the stressed animals, either alone or with RES, caused a significant increase in LAE and decrease in BI (*Fig. 3*) indicating the reduction of the depression-like behavior in FST with FLX treatment. The reduction of depressive behavior in CUMS-exposed rats with FLX, as indicated by increased sucrose intake (*Fig. 1*), as well as decreased BI and increased LAE in FST (*Fig. 3*) may be related to the enhanced 5-HT action.

Stress is considered a major factor contributing to male infertility. Several workers noted that stress significantly reduced sperm count and motility (37). Moreover, severe stress of a chronic or intermittent duration can have detrimental effects on reproductive processes in animals. In a study by Almeida *et al.* (6), chronic immobilization stress of male rats from prepuberty to sexual maturity caused a significant decrease in Ts secretion. The inhibitory effect of chronic immobilization on plasma Ts has been a common finding in adult rats attributed to reduced LH concentration (38). The precise mechanism of the inhibitory effect of prolonged stress on plasma Ts, however, is not fully understood since the changes in androgenic response are not always associated with altered LH levels (39). It was suggested that restraint stress may induce Leydig cell hyposensitivity to gonadotropin (Gn) leading to a blockade of Ts biosynthesis at normal LH levels (40). Immobilization stress also provokes an elevation of serum corticosterone concentrations which causes the decline in Ts concentrations (41). In the present study, similar hormonal changes were observed during CUMS as indicated by decreased plasma Ts level in CUMS-exposed rats (*Table 1*). It is postulated that during stress, increased corticosteroids suppress Gn and directly suppress testicular function (42). In addition, stress-mediated increases in corticotrophin-releasing factor (CRF) result in a reduced secretion of the Gn-releasing hormone (GnRH). It has been suggested that CRF acts directly by suppressing GnRH or indirectly by increasing opioid release (43). Other data have shown that increased glucocorticoids may act *via* glucocorticoid receptors on Leydig cells, thus suppressing the testicular response to gonadotropins (37). The proopiomelanocortin (POMC)-derived peptides may also play an autocrine/paracrine role in mediating the stress-induced decline in testicular steroidogenesis (44). Furthermore, increased serum corticosterone have been reported to induce Leydig cell apoptosis and impair Leydig cell steroidogenesis (45). Also, physiological levels of leptin stimulate steroidogenesis and follicle maturation, whereas supraphysiological concentrations of leptin may produce the opposite effect (46).

A significant decrease in both the production of maturing spermatids and concentration of spermatozoa in the cauda epididymidis was observed in stressed rats (6). The decreased androgenic status of stressed rats may be responsible, at least in part, for depressing spermatogenesis. Also, local regulatory interactions occur between all testicular cell types (interstitial, peritubular myoid, germ and Sertoli cells) and these cell-cell communications, involving growth factors and POMC peptides, mediate the cell growth and differentiation required for the initiation and maintenance of spermatogenesis. The stressful stimulus may have interfered with elements of this elaborate paracrine control, impairing the spermatogenic process (6). Moreover, Kim *et al.* (47) demonstrated that Ts withdrawal induces spermatocyte apoptosis *via* caspase-3 activation and caspase-activated deoxyribonuclease expression. In addition, a transient reduction of serum Ts caused by immobilization stress can enhance testicular germ cell apoptosis in rats (48). In the present study, similar changes in the sperms were observed during CUMS. Plasma Ts and sperm count and motility decreased while abnormal sperms increased in CUMS exposed rats (*Table 1*). FLX treatment of CUMS rats caused a further decrease in plasma Ts, sperm count and motility and increase in abnormal sperms (*Table*

1). These results suggest impairment of spermatogenesis which may be explained by decreased Ts level observed in CUMS and CUMS + FLX rats (*Table 1*). RES administration to stressed rats, both alone or with FLX, increased the plasma Ts, sperm count and motility and decreased the abnormal forms (*Table 1*). Therefore, while FLX improved the depressive-like behaviors in CUMS rats, it worsens the testicular dysfunctions. These deteriorating effects of FLX are ameliorated by co-administration of RES.

Sexual dysfunction as loss of libido, impaired orgasm, delayed or absent ejaculation, decreased testicular development and decreased Sertoli cell population are possible side effect of antidepressant therapy as FLX which may lead to infertility in adults (49, 50). In a study by Kumar *et al.* (51), they demonstrated that FLX, sertraline, fluvoxamine, and citalopram negatively influence semen parameters and showed a spermicidal effect. In addition, long-term intake of FLX caused a decrease in spermatogenesis, weights of reproductive organs and Ts and FSH levels in rats (9). Testicular damage, caused by FLX, was associated with increased MDA and reduced GSH (9). The decrease in Ts may result from action of FLX on Leydig cells or effect of ROS on testicular steroidogenesis (9). Also, chronic FLX administration caused liver injury through inducing oxidative stress (52). Moreover, its use was accompanied by other side effects as lung damage (53), hepatotoxicity (54) and cardiotoxicity (55). In accord with these observations, the present study demonstrated that FLX treatment of CUMS rats caused a decrease in plasma Ts, sperm count and motility, and increase in abnormal sperms (*Table 1*).

Oxidative stress develops in association with an imbalance between ROS and the antioxidant reserve system. ROS are the product of normal cellular metabolism. Antioxidant defense mechanisms in the testis such as various antioxidants, vitamins, and glutathione are important in the protection of sperm against ROS (56). In the present study, we evaluated the level of lipid peroxidation product (MDA) and anti-oxidants (GSH, SOD and catalase) in the testicular tissue of rats exposed to CUMS-induced depression with or without FLX and RES administration. Our results showed a significant increase in MDA and decrease in anti-oxidants (GSH, SOD and catalase) in the testis of rats exposed to CUMS (*Table 2*) which clearly confirms the presence of testicular oxidative stress in the depressed rats. FLX administration to CUMS rats further worsens the testicular oxidative stress as indicated by the significant increase in MDA and decrease in anti-oxidants (GSH, SOD and catalase) as compared with untreated CUMS animals. RES either alone or with FLX significantly decrease MDA and increased anti-oxidants (GSH, SOD and catalase) in the testicular tissue of rats exposed to CUMS (*Table 4*). The results of the current work were in accord with those of previous studies (57). Additionally, one study showed that RES can serve like the antioxidant enzymes SOD and glutathione peroxidase (58). The protective effect of RES treatment may be due to its protection of cellular membranes against oxidative damage (59).

The role of oxidative stress in the development of testicular dysfunction under stress induced depression is not well understood. The sperm cell is distinct from other germinal or somatic cells in its ability to generate ROS as well as its great vulnerability to such molecules (60). With ROS production at low levels, sperm cell capacitation, acrosome reaction, and sperm binding to the zona pellucida take place whereas uncontrolled ROS production leads to sperm abnormalities and infertility (61). In previous related human studies it has been shown that oxidative stress is associated with a reduction in sperm motility, viability and defects in sperm-oocyte fusion (62). Moreover, under an elevated oxidative stress status, ROS cause cellular injury *via* several mechanisms including lipid peroxidation and oxidative damage of proteins and DNA (52). Either pathways of oxidative stress, direct oxidative damage to genomic DNA or up-regulate apoptotic proteins, leads to germ cell death and impaired spermatogenesis (63). In consistent with these findings, the results of the current work showed a significant decrease in sperm count and motility and increase in the abnormal forms in CUMS-exposed group with a further deterioration by FLX treatment (*Table 1*). RES administration to those CUMS-exposed rats, both alone or with FLX, increased the sperm count and motility and decreased the abnormal forms (*Table 1*). Therefore, the decreased sperms count and motility and increased abnormal sperms in CUMS-exposed rats may be related to the adverse effects of testicular oxidative stress on spermatogenesis. In addition, the worsening effect of FLX on testicular oxidative stress in CUMS rats may lead to a further decrease in sperms count and motility and increased abnormal sperms.

There is increasing evidence that functionality of steroidogenic tissues might be compromised in states of persistent oxidative stress with generation of ROS. A number of studies with rat primary testicular Leydig cells showed that levels of steroidogenic enzymes declined with advancing animal age along with Ts output in parallel with diminished levels of antioxidants (64). Leydig cells are reported to be particularly susceptible to oxidative damage *in vivo* due to their close proximity to ROS-producing testicular interstitial macrophages (65). Omura and Morohashi (66) reported that ROS such as superoxide anions and H_2O_2 could inhibit testicular steroidogenesis. In accord with these studies, the results of the current work demonstrated a significant decrease in plasma Ts level in CUMS-exposed rats with further deterioration by FLX treatment (*Table 1*). Moreover, RES significantly increased plasma Ts level in CUMS animals with or without FLX administration (*Table 1*). In addition, a negative correlation between MDA and Ts level ($r = –0.8750$; $P < 0.001$; $n = 50$) was reported (*Fig. 4*). Therefore, these results denote that the decrease in Ts in CUMS exposed rats with further reduction by FLX treatment may be related to the state of oxidative stress developed in those rats. This decrease in Ts level may be responsible for decreased sperm count and motility and increased abnormal forms (*Table 1*).

In order to determine the likely mechanism of action of stress-induced depression, FLX and RES on Ts levels, we analyzed the mRNA expression level of two key steroidogenic proteins, namely StAR and P450scc. StAR is a protein that mediates the rate-limiting step of steroidogenesis. StAR participates in the transport of substrate cholesterol from outer to inner mitochondrial membrane in Leydig cells, where cytochrome P450scc is located. The first enzymatic step in steroidogenesis is the conversion of cholesterol to pregnenolone, catalyzed by cytochrome P450scc enzyme (5). The results of the present study showed a significant decrease in the StAR and P450scc gene expression in rats subjected to CUMS-induced depression with more lowering by FLX administration (*Fig. 5*), therefore explaining the decreased Ts level in CUMS-exposed rats with further reduction by FLX treatment. Previous studies demonstrated that the inhibition of StAR protein by ROS is a major target of ROS attack in rat Leydig cell culture, thus reducing steroidogenic potency of these cells (7). The results of the current work support these studies as shown by the significant increase in StAR and P450scc gene expression in CUMS-exposed rats administered RES either alone or with FLX (*Fig. 5*) which suggest the involvement of oxidative stress in the inhibition of StAR and P450scc gene expression in those rats. Thus, we could explain the increased Ts level in CUMS-exposed rats administered RES with or without FLX by the increased StAR and P450scc gene expression in those rats as a result of the anti-oxidant action of RES that attenuates the state of oxidative stress developed in those rats.

Our histopathological findings support the biochemical results. The CUMS-exposed group had a lower testicular Johnson's score than the control group with a further decrease by

FLX treatment (*Table 3*). Widespread immature degenerated germ cells with vacuolization of epithelium, damaged Sertoli cells and necrotic Leydig cells were present in the seminiferous tubules of the CUMS-exposed rats with or without FLX (*Fig. 6B* and *6C*). This shows that spermatogenesis was not completed and was impaired. These alterations may be due to oxidative stress that developed in CUMS-exposed rats with or without FLX. In addition, histological examination of testicular tissue in stressed rats administered RES with or without FLX showed marked improvement in the histopathological changes induced by CUMS (*Fig. 6D* and *6E*). In addition, Johnsen's score was significantly increased in RES treated groups. These results therefore indicate that the oxidative stress, provoked by CUMS-induced depression or FLX, has a key role in initiating tubular damage in the testis. Also these results confirm the protective effect of RES.

In summary, RES treatment has no significant effects on behavior deficits, neurotransmitters and corticosterone induced by CUMS, while it protects testicular functions. Fluoxetine improves depression-related behaviors, but it damages sperm activities. Moreover, resveratrol protects rats from CUMS-induced oxidative stress, while fluoxetine further worsens it, as indicted by the measurement of SOD, MDA, CAT, GSH. So, this article shows the different effects of RES and FLX on depression, testicular functions, and oxidative stress. The combination of RES and FLX was more effective compared with stress group, but no significant improvement was obtained when compared with the better one used separately. So the value here is the alleviation of the side effects caused by antidepressant treatment of FLX.

In summary, the CUMS-induced depression decreases sucrose intake (anhydonic behavior) and weight gain, induced testicular oxidative stress and caused testicular dysfunctions in rats. While treatment of depressed rats with FLX increases sucrose intake and weight gain, it worsens the testicular oxidative stress and testicular dysfunctions. Finally, administration RES improves the testicular oxidative stress and testicular dysfunctions caused by CUMS-induced depression and worsened by FLX administration. Therefore, RES could be given with FLX for treatment of depression as it improves the testicular oxidative stress and testicular dysfunctions caused by FLX treatment.

Conflict of interests: None declared.

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