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Behavioral and neurochemical changes induced by post-weaning female rats isolation

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Abstract: Rodents post-weaning isolation could be considered a significant early-life model of chronic stress. The aim of our study was to investigate the effects of female rats post-weaning isolation on different behavioral tests and neurochemical parameters. On postnatal day 21, female pups were housed either isolated or socially in groups (4/cage) for 6 weeks. After the 6th week adult female rats were behaviorally examined using open field test, three chamber social interaction test, hole board test and condition avoidance test. In addition, hippocampal content of neurotransmitters, oxidative stress and inflammatory markers were measured. Isolated female rats exhibited behavioral changes, female rats shows a significant increase in anxiety and a significant decrease in locomotor activity in comparison to the control group using open field test. Moreover, female rats isolation promoted a significant decrease in sociality index as compared to control groups. Finally, female rats post-weaning isolation of invoked a significant impairment in long-term and short-term memory evaluated by both conditioned avoidance and hole board tests, in comparison to the control group. Furthermore, female rats post- weaning isolation induced oxidative stress, inflammation, and neurotransmitter alterations. Collectively female rats post weaning isolation had significantly prompted both behavioral and neurochemical changes which could represent the essential important symptoms of schizophrenia.

Keywords: Isolation; Dopamine; Hippocampus; Oxidative Stress Markers; Inflammatory Markers.

1. INTRODUCTION

Neuropsychiatric disorders covered up a huge diversity of symptoms which are the main causes of morbidity and disability. Generally, the psychiatric disorders prevalence was estimated to be 6.7%.[1] A lot of studies have been performed to clarify the molecular mechanism which is responsible for occurrence of such neuropsychiatric diseases. One of the most serious and terrifying Neuropsychiatric disorders is Schizophrenia, it is ranked among the top ten illnesses that contribute to the global burden of disease according to world health organization .[2]

Schizophrenia is a chronic psychotic disorder, which is characterized by positive, negative and cognitive symptoms. It causes a mess up of the patient thoughts and affects its ability to engage in social interaction. No other disorder arouses as much anxiety as schizophrenia.[3] One of the most difficult challenges facing us is the complex nature of schizophrenia,

which makes understanding of the etiology and pathogenesis of schizophrenia and developing new more effective treatments critical.[4] Numerous researches had shown that early-life experiences can affect adulthood behavior.[5] This outcome is very significant because in such a way genetics had become not the only factor that influences adulthood behavior.[6]

It had been suggested that social isolation and loneliness in childhood period had a harmful impact on an area of the brain that regulates social behavior in adulthood. [7] These behavioral changes may be sex related, as a number of researchers suggested that loneliness was more robustly linked with depression symptoms elevation in females and with social anxiety elevations in males.[8,9] Loneliness period length appears to be an alert of upcoming mental health troubles.[10] Researchers suggested that social isolation during childhood period, teenagers and early

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adulthood appears to augment individuals risk for future schizophrenia-spectrum disorders development. They suggested that parents early support and encouragement for their children to increase positive social interaction during their early life will help them greatly to overcome social anxiety and stress in the future.[11] The animal modeling of loneliness is carried out via the social withdrawal of rat pups since their weaning age and raising them in separate cages away from the other pups. This model is called post-weaning social isolation. The defined social structure and developed hierarchy exhibited by rats within a colony play an important impact on animals neurobiologicaland behavioral development, that are unaffected with social re-integration during their later on life. Thus, post-weaning isolation alters development of the brain causing behavioral impairment during adulthood.[12] These behavioral deficits represents some of schizophrenia essential.[13] The isolation model has a high appealing as it has quietly uncomplicated procedure to be carried out, but this model requires enormous spaces for housing all individual cages. This model usefulness is in mimicking some neurochemical, behavioral and neuropathological characteristics of schizophrenia disorder.[14] Therefore, the present study was conducted to investigate the effects of post-weaning social isolation of female rats on diverse of behavioral and neurochemical parameters.

2. METHODS

2.1. Experimental animals

Our study was accomplished according to the use and care of laboratory animals guide declared via the national institutes of health (NIH publications no 23, revised 1978) and the committee of ethics of pharmacy faculty (girls), Azhar University. Twenty-four Sprague-dawely female were received just after weaning from their mother rats (21 days after birth with initial weight 21-25g). Female rats pups were purchased from the National Research Institute, Cairo, Egypt. Weaned female pups were reared either grouped in cages or isolated in departed cages following normal conditions (24±1ºC and 12-hrs dark-light cycle). water and food were available freely. Before any experimentations female rats had 7 days acclimation.

2.2. Experiment design

Twenty-four female pups were divided randomly to two groups, each one of 12rats.

First group: control rats; weaned pups were socially reared in same cages (4/cage) and served as control.

Second group: isolated-reared rats; weaned pups were isolated in separate cages for 6 weeks.[15] During the last 4 days of the 6th week of isolation, behavioral studies were performed then we sacrificed the rats and dissected the hippocampal tissues, immediately we rinsed them with cold saline and blotted them with filter paper. We homogenizes hippocampus tissues using saline to be further used in analysis. Hippocampal protein content was measured in the homogenates via standard bovine serum albumin using Bradford technique.[16]

2.3. Behavioral assessments:

Four different behavioral tests were carried out:

2.3.1. Open-field test

This test represents a mild condition of stress [17] and is considered as a common test for locomotor activity, emotionality, excitability and exploratory aspects in rats.[18] The open field device is made up of wooden box (40cm x 80 cm x 80 cm),[19] having white floor and 4 red sides. Black lines divided the field floor into 16 equal small squares (4 x 4). This experiment was conducted in a quiet room.

Procedure

An hour before the test, rats were weighed, and all female rats were transferred to the test room after water and food removal from animal cage. Rats were taken alternatively and placed in centrally in the open field box and recorded for 3 minutes. We scrubbed the walls and floor following each tested female rat and then returned to their cages. The female rat behavior in the open field box was recorded throughout the 3 minutes (period of observation) to calculate the following parameters: latency time, ambulation frequency, rearing, grooming and pellets count.[17,19]

2.3.2. Three-chamber social interaction test

It is conducted for the study of social affiliation in rats.[20] The apparatus consists of glass 3 chamber rectangular box. The dimension of the chamber is 45x19 cm with separating walls made of transparent Plexiglas. The dividing wall has a middle opened section, that permits the entrance to the chamber. Each chamber has a wire cup-like containers placed vertically inside the apparatus. The experiment was carried out in a quiet room. We use two types of rats, first one acts as a control and the other is the test subject.

Procedure

In the beginning the sides compartments are isolated by Plexiglass walls and the subject rat is placed in the middle chamber center adapted for 5 min. After that control rats is placed in the wire cup which is placed in one of the side chambers then the walls in-between compartments were detached to permit

subject rat entrance in order to explore the three chambers for a period of 10 min. Time spent in stranger's zone and around its cup was measured versus time spent in empty chambers to estimate Sociability index (SI). Decrease in sociality index indicates social withdrawal.

2.3.3. Hole board test

This test was conducted in accordance with Galey and Jaffard's method[21] and which was modified via Schroeder et al. in order to assess the short-term memory performance.[22] The hole board box was made of wood (40 cm x 30 cm x 30 cm), having 3 black side walls and the 4th one was made of glass. The floor is black having 4 holes that contain food cups and located 8.5 cm apart from the apparatus walls.

Procedure:

All experimental animals were maintained at 80% of the consumed usual food consumed for 3 consecutive days before the experiment day. During this partial food insufficiency phase, every animal remained for 10 minutes daily in the apparatus (3 repeated days). Water and food were removed at the night prior to the experiment. The experimental animals were weighed and transfer to test room an hour prior to the test. The test was conducted in a quiet room. Firstly, experimental rat was positioned in the apparatus (facing the transparent wall) for two minutes with the availability of five pellets in certain hole (Acquisition 1). Subsequently, the female rat was returned back for one minute in its cage, then it was placed once more in the apparatus for 2 minutes with pellets of food placed in an neighboring hole (Acquisition 2). We carried out a Retention test for five minutes after acquisition 2 trial and for 3 minutes without any pellets in the apparatus. We evaluated retention test by recording the "visited holes" numbers containing food during the two acquisitions and percentage of correct response is calculated.[22]

2.3.4. Conditioned-avoidance test

This procedure was carried out according to Arnt[23] as well as the modification adopted after Garofalo et al.[24] It is a wooden box 11.7x 43.8x 15.6 inches, divided by movable glass panels into 5 inter-connected chambers. The 4 chambers floors were manufactured of parallel metal rods grid. While the 5th chamber's floor was produced of glass. The 4 chambers' floors grid was electrified using a simulator, that was adjusted at 25 pulses/sec and 50 volt. The 1st electrified chamber had an electric bell, which was fixed in it.

Procedure

The experimental animals were weighed and taken to the test room an hour prior to the test and after removing water and food from their cages. The experiments were conducted in a calm room. The tested rats were taken alternatively from random cages. Animals were alternatively trained in the apparatus. The parameters were manipulated to evaluate memory and learning ability under stressful conditions. The training of the rats was performed by coupling of auditory stimulus for five sec. then a foot shock for another five sec. For each one of the rats we calculated the trials number [at first and second days] to arrive at the safe area (to stay away from the electric shock) during five sec. of the auditory stimulus prior to the electric shock starting.

2.4. Assessment of dopamine level in rats hippocampus

The level of dopamine in hippocampus of female rats was determined using commercially available ELISA kits of CUSABIO (USA) catalog number (CSB-E08660r). This ELISA assay applies the competitive immunoassay technique utilizing polyclonal anti-dopamine antibody and dopamine-horseradish peroxidase conjugate. The results were expressed as ng dopamine/ml.

2.5. Assessment of serotonin receptors-2A level in rats hippocampus

The level of sertonine receptors-2A ($5HT_{2A}$) in hippocampus of female rats using the competitive ELISA techniqueutilizing a polyclonal anti-5-HT_{2A} antibody and 5-HT_{2A}-HRP conjugate. We used was commercially available ELISA kits of CUSABIO (USA) catalog number (CSB-E14956r). we expressed our results as pg $5HT_{2A}$ receptors/ml.

2.6. Hippocamal oxidative stress markers assessment

Glutathione (GSH) was measured in accordance to Ellman mehod.[25] In which, 0.5 ml of the hippocampal homogenate were placed in a test tube with 0.5 ml of 10% trichloroacetic acid. The lipid peroxidation was assesed by determining thiobarbituric acid reactive substances level measured in terms of malondialdehyde (MDA), in accordance to Mihara and Uchiyama method.[26] The results of MDA were represented as nmol MDA/g wet tissue. The superoxide dismutase (SOD) activity was estimated using ready made available kits following the manufacturer's instructions (Bio-diagnostic, Egypt). SOD are metalloenzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide and thus form a crucial part of the cellular antioxidant defense mechanism. This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye. The results were expressed as U SOD/g tissue.

Parameters	Control group	Isolated group
Latency time (sec.)	2.67 ± 0.82	1.5 ± 0.9[a]
Ambulation frequency (squares/3min)	20.2 ± 4.5	34.7±4.9[a]
Rearing (counts/3min)	7.5 ± 1.38	10 ± 1.1[a]
Grooming (cunts/3min)	1.5±0.84	3.34±1.03[a]

2.7. Assessment of inflammatory markers in rat hippocampus

ELISA kits of CUSABIO (USA) were used to evaluate tumor necrosis factor-α levels (TNF-α) catalog number (CSB-E11987r). This kit is based on sandwich ELISA technique, where Anti-TNFα polyclonal antibody was pre-coated onto 96-well plates and the biotin conjugated anti- TNFα polyclonal antibody was used as detection antibodies and the final results were expressed as pg/mg protein.

Nuclear factor-kappa B p65 levels (NF-κB p65) was measured using ELISA kits of CUSABIO (USA) catalog number (CSB-E13148r). the principle of the assay employs the quantitative sandwich ELISA technique in which, a monoclonal antibody specific for rat NF-κB has been pre-coated onto a micro plate and the biotin conjugated anti- NF-κB polyclonal antibody was used as detection antibodies. The final results were represented in pg/mg protein**.**

2.8. Statistical Analysis

The results were expressed as mean values ± standard deviation (SD). The statistical analysis was conducted using unpaired t-test. *P*-values less than 0.05 were regarded as indication for statistically significant differences between groups compared. All statistical analyses were made and graphs were plotted via GraphPad Prism (ISI®, USA) software (version 5).

3. RESULTS

3.1. The behavioral assessments:

3.1.1. The open field test

The open field test is considered as a frequent test for locomotor activity, emotionality, excitability and exploratory aspects in rats. Female rats post-weaning isolation for 6 weeks significantly decreased the latency time by 46% in comparison with the control group which indicated that social isolation increased excitability. Moreover, isolation increased the exploratory activity significantly which was indicated by increased ambulation frequency to 72% and frequency of rearing to 36% as compared to the control group. In addition, as an emotionality manifestation, self-grooming was increased significantly in the IR group by 107% in comparison with the control group, however Number of pellets was not significantly affected (**Table 1).**

Table 1: Effect of female rats post-weaning isolation for 6 weeks on the behavioral changes assessed by the open field test.

Data are expressed as mean ± SD (n=6). [a]: Significantly different from corresponding control group, respectively at P <0.05 using unpaired t-test.

3.1.2. The three chamber social interaction test

Here we tested the effect of social isolation of female rats on the social interaction of the rats with each other. It was clear that isolation of female rats for 6 weeks induced social withdrawal manifested by a significant decrease in sociality index (SI) by 63 % in comparison with control group (**Figure 1).**

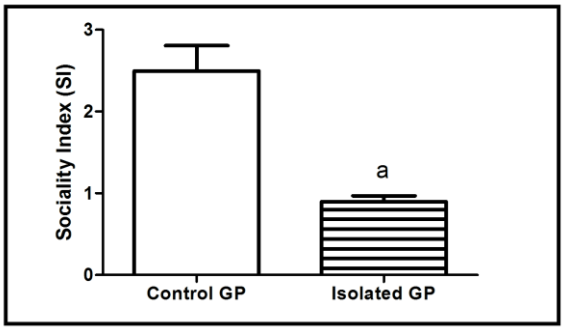

Figure 1: Effect of female rats post-weaning isolation for 6 weeks on sociality index (SI) assessed by the three chamber social interaction test**.** Data are expressed as mean ± SD (n=6). [a]:Significantly different from corresponding control group, respectively at P <0.05 using unpaired t-test.

3.1.3. The hole board test

Figure 2 illustrated that isolation of female rats significantly decreased the short-term memory measured as % of correct response by 42%, in comparison with the control group.

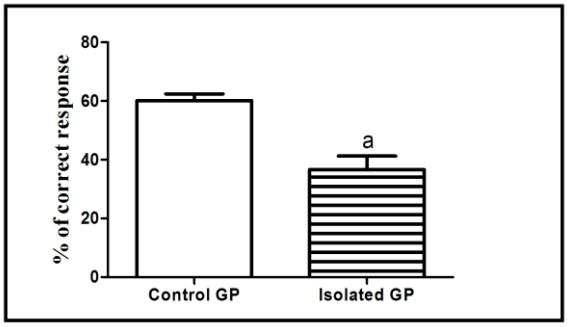

Figure 2: Effect of female rats post-weaning isolation for 6 weeks on short-term memory assed by the hole-board test..Data are expressed as mean ± SD (n=6). a: Significantly different from corresponding control group, respectively at P <0.05 using unpaired t-test.

3.1.4. The conditioned-avoidance test

Female rats post-weaning isolation for 6 weeks induced a significant impairment of learning measured in terms of increased trials number to stay away from electric shock at the experiment first day, by 53%, compared to control group (**Table 2**). Moreover, female rats isolation induced an long-term memory impairment represented as an increase in trials number significantly to keep away from the electric shock at the experiment second day, by 173%, in comparison to control group (**Table 2**).

Table 2: Effect of female rats post-weaning isolation for 6 weeks on long term memory assessed by the conditioned avoidance test.

Parameters	Control group	Isolated group
First day	4.33±2.1	7.33±1.64[a]
Second day	1.5±0.8	4.3±1.5[a]

Data are expressed as mean ± SD (n=6). **[a]**: Significantly different from corresponding control group, respectively at P <0.05 using unpaired t-test.

3.2. Assessment of dopamine level in the hippocampus

Post weaning social isolation of female rats for 6 weeks induced an increase in the hippocampal dopamine content significantly by 158%, compared to control group (**Figure 3**).

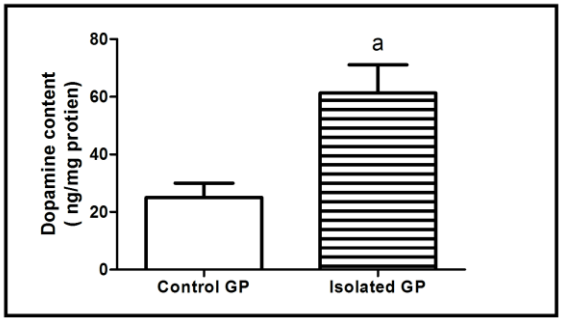

Figure 3: Effect of female rats post-weaning isolation for 6 weeks rats on the hippocampal dopamine level.Data are expressed as mean ± SD (n=6). **[a]**: Significantly different from corresponding control group, respectively at P <0.05 using unpaired t-test.

3.3. Assessment of serotonin receptors-2A level in the hippocampus

Figure 4 showed that female rats isolation for 6weeks induced a significant increase in the hippocampal $5HT_{2A}$ receptors content by 168.8%, in comparison with the control group.

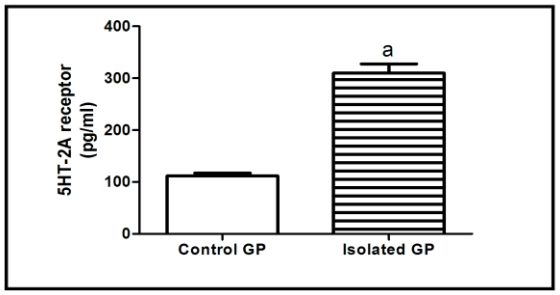

Figure 4:. Effect of female rats post-weaning isolation for 6 weeks on hippocampal serotonin receptor-2A level. Data are expressed as mean ± SD (n=6). [a]: Significantly different from corresponding control group, respectively at P <0.05 using unpaired t-test.

3.4. Hippocamal oxidative stress markers assessment

Female rats post-weaning social isolation induced redox imbalance was confirmed by evaluating GSH, MDA and SOD levels in rat hippocampal tissue. Table 3 showed that post-weaning social isolation reduced GSH levels significantly by 69% and increased lipid peroxides level significantly by almost 4 folds compared to control group. Besides, post-weaning social isolation induced a decrease in the hippocampal SOD levels significantly by 76%, in comparison with control values (**Table 3**).

Table 3: Effect of 6 weeks of post-weaning isolation rearing of female rats on hippocampal oxidative stress markers and antioxidant enzymes activity.

Parameters	Control group	Isolated group
GSH (mmol/g tissue)	91.12±7.4	27.75±4.1[a]
MDA (nmol/g tissue)	12.7±1.3	57.8±6.5[a]
SOD (U/g tissue)	4.4±0.33	1.04±0.11[a]

Data are expressed as mean ± SD (n=6). a: Significantly different from corresponding control group, respectively at P <0.05 using unpaired t-test.

3.5. Assessment of inflammatory markers in rat hippocampus:

It was obvious that female rats post-weaning social isolation induced an elevation in TNF-α level significantly by 347%, in comparison with the control group (**Figure 5A**). In addition, female rats post-weaning isolation showed an elevation in NF-κB level significantly by 156% in comparison to the control group (**Figure 5B**).

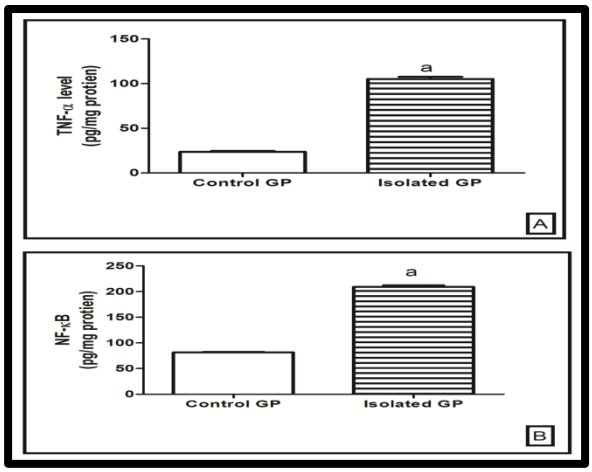

Figure 5: Effect of female rats post-weaning isolation for 6 weeks on hippocampal inflammatory markers: (**A**) Tumor necrosis factor alpha (TNF-α); (**B**) Nuclear factor kappa B (NF- κB). Data are expressed as mean ± SD (n=6). [a]: Significantly different from corresponding control group, respectively at P <0.05 using unpaired t-test.

4. DISCUSSION

Female rats Post weaning isolation is a widespread model that is used to study different early-life stress effects on later brain and behavior activity.27 Particularly, rats isolation rearing most potent effects take place during the critical phase from weaning to just before adulthood.28 Post-weaning social isolation, being a chronic stress model, is documented to be one of the major risk factor for a lot of psychiatric diseases such as schizophrenia.[29] In the current study, we carried out a pilot study on isolated reared female rats to assess the behavioral and neurochemical alterations occurred subsequent to 6 wks of post-weaning isolation to ensure induction of schizophrenic-like symptoms before trying new remedies. That's why we selected post-weaning social isolation for induction of pathophysiological and behavioral changes that are reliable with schizophrenia core symptoms.[30]

Firstly, the rats were tested by the open field test to evaluate schizophrenia positive symptoms, then they were tested by the three chambers social interaction device to judge schizophrenia negative symptoms and finally we tested the female rats using the conditioned-avoidance test and the hole bored test to evaluate schizophrenia cognitive symptoms.

As regard the open field test, it was obvious that female rats post weaning isolation induced a significant increase in locomoter behavior manifested as decrease in latency time and increase in the ambulation frequency, in accordance with earlier study.[31] Moreover, isolation of female rats for 6 weeks induced anxiety in the open field test manifested as increase the frequency of grooming, as previously observed .[32, 33]

In addition, female rats post weaning isolation induced social withdrawal manifested by significant decrease in sociality index evaluated by the three chambers social interaction test.[34] The isolated reared female rats induced a significant decrease in short term memory, long-term memory and learning impairment, which were evaluated by both conditioned avoidance and hole board tests. This come in accordance with the preceding study that showed that long-term social isolation of females rats compared to males ones showed more obvious cognitive deficits in morris water maize as well as in the passive avoidance paradigm.[31]

In parallel with the enhanced locomotion in open field test, female rats post weaning isolation showed elevation in the hippocampal dopamine content, illustrating the importance of dopamine in the schizophrenia pathophisolgy.[35] In general, elevation of the hippocampal dopamine content has been documented in patients of schizophrenia previously.[36]

Moreover, female rats post-weaning isolation induced elevation of the hippocampal 5HT2A receptors content. Serotonin 5-HT2A receptors are extensively scattered in different brain regions and they are essential for learning and cognition. Abnormal 5HT2A receptors activity is linked with numerous psychiatric diseases such as schizophrenia, depression and drug addiction.[37] Disruption of the neurotransmitter content could illustrate the behavioral abnormalities evoked by female rats post-weaning isolation.

In order to clarify the different mechanisms by which post-weaning isolation of female rats induced behavioral changes, we assessed oxidative stress markers. Oxidative stress has an essential role in injury of brain neurons and schizophrenia pathophysiology.[38] During condition of stress, the oxidative injury occurs as a result to huge content of reactive oxygen species produced by mitochondria that correlates with ATP and GSH depletion. [39-40] Moreover, it was recognized that oxidant/antioxidant equilibrium imbalance occurs as a result of production of NO and ROS in brain's certain regions such as hippocampus during the exposure to psychosocial stressors. [41] In our study, the elevated MDA level along with the reduction of both GSH and SOD level confirmed the induction of oxidative stress as a result of post weaning isolation of the animals, this comes in agreement with earlier studies.[42,43]

Chronic isolation of female rats disrupts brain redox homeostasis and debilitates its antioxidant defenses as previously confirmed through numerous study.[44,45] As a result, BDNF transcription factor (NF-κB) becomes very sensitive and active.[46] NF-κB has been concerned to play a critical role in neuropsychiatric disorders pathogenesis, such as

depression and schizophrenia.[47] In agreement with previous studies, female rats post weaning isolation promoted a significant elevation in hippocampal NF-κB and TNF-α levels.[48, 49] It is obvious that social isolation for rats spaced out from another treatments aggravate social-isolation-induced stress behavioral depression associated with excessive changes in anti-inflammatory plasma cytokines.[49]

5. CONCLUSIONS

Post weaning isolation of female rats induced significant behavioral and biochemical changes that could represent schizophrenia essential symptoms and shall be considered as suitable animal model used for induction of schizophrenia.

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Conflicts of Interest: The authors declare no conflict of interest.

Ethical Statement: The protocol used in this study was approved by the Al-Azhar Faculty of Pharmacy (Girls) animal ethics committee (no 80,2016).

Author Contribution: This work was carried out in cooperation between all authors. AG. and WE designed the study. AG carried out the experiments. AG and WE wrote the manuscript; WE, EE, and AA analyzed data and revised the manuscript.

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