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Therapeutic Efficacy of Adipose-Derived Mesenchymal Stem Cells after Chronic Fluoxetine Treatment on pars nervosa in adult albino rats (light and immunohistochemical study)

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Abstract

Background: Fluoxetine (FLU) is a prototype of SSRIs and a first line drug for the treatment of depression. **Aim of the work**:This study was planned todemonstrate the histological features of pars nervosa after chronic fluoxetine treatment and the possible therapeutic effect of adipose-derived mesenchymal stem Cells.**Material and Method:** Thirty healthy male adult albino ratswere classified into four groups. Control group(Group I) included fifteen rats. Fluoxetine treated (Group II) included five rats that received 24 mg/kg/day of fluoxetine dissolved in 1.0 ml of tap water once a day. Fluoxetine group treated with ADSCs (Group III)included five rats that received fluoxetine as group (II) for30 days then injected once by ADSCs at a dose of 1×10^6 cells/rat in the tail vein suspended in 0.5ml of phosphate-buffered saline (PBS). Recovery group (Group IV) included five rats that received fluoxetine for 30 days then received no treatment till the end of the experiment. Samples from pars nervosa were processed for light,immunohistochemical, morphometrical and statistical analyses. **Results:** Fluoxetine treated group showed pituicytes with different shaped nuclei and pale or vacuolated cytoplasm. Acidophilic small Herring bodies were noticed. Administration of ADSCs greatly improved microscopic appearance ofcellswhile, recovery group showed more or less similar histological changes as Fluoxetine group. **Conclusion**: Fluoxetine induced various deleterious changes on the pars nervosa of albino rat. These changes were almost corrected by ADSCs treatment.

Key words: fluoxetine, pars nervosa, Adipose-Derived Mesenchymal Stem Cells, rats, S100 protein.

Introduction

Right now, up to 121 million people worldwide suffer from major depressive disorder (MDD). The prevalence is rising and it is expected to represent the second most prevalent disorder in the world. There have several reports for the effect of fluoxetine on hormonal axis of pituitary-gonad in rats. These studies have also shown that it has interaction with sex hormones in both sexes **[1].** Also, the histopathological effects of fluoxetine on vital organs; kidney and liver have been assessed **[2], [3].**

In recent decades, the biomedical applications of stem cells have attracted increasing attention. Many reports showed that ADSCs-based cell therapy products demonstrated optimal efficacy and efficiency in some clinical indications for both autologous and allogeneic purposes. Hence, they are considered the most attractive stem cell source for tissue engineering and regenerative medicine **[4].**

The family of S-100 protein has arole in the regulation of proliferation, differentiation, apoptosis, energy metabolism, inflammation and migration/invasion of many cells. S-100 is a good marker for supporting cells in the pars nervosa **[5].**

Studies still few about the effect of transplanted ADSCs on pars nervosa function and structure. So, this study was destined to detect the therapeutic potential of ADSCs on pars nervosa alternations induced by chronic fluoxetine treatment in rats using histological approaches.

Materials and Methods

Animals

Thirty healthy adult male albino rats were utilized. They were received and kept according to the guidelines of animal house, Faculty of Medicine, Zagazig University **(#4080, Revised 10/2017).**

Chemicals and preparation

Flutin (Fluoxetine hydrochloride):it was obtained in the form of capsules; each capsule contains 20 mg of Fluoxetine hydrochloride (EIPICO Company, Egypt).Fluoxetine was dissolved in 1.0 ml of tap water (as a vehicle).

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Adipose-derived mesenchymal stem cells (ADSCs):
Adipose-derived mesenchymal stem cells labeled with Paul Karl Horan 26 (PKH- 26) (red fluorescence cell linker) were obtained from Kasr Al-Ainy Medical School, Biochemistry Department.
Isolation and characterization of ADSCs: Adult male albino rats 6weekold were anesthetized with xylazine (15 mg/kg) and ketamine (85 mg/kg) injected intraperitoneally. Adipose tissue was harvested from the upper part of the intestine, then chopped to the small pieces and digested in an incubator with 0.02 mg/ml collagenase type I for 1 hour. The suspension was centrifuged for 5 minutes and the cell pellet was separated **[6].**
The sample ADSCs was transferred to the culture medium consisted of DMEM supplemented with 10% fetal bovine serum (FBS), streptomycin and penicillin in a humidified incubator. After 24 hours, non-adhered cells were removed. The Cell cultivation was preserved up to the third passage and cells were then characterized by differentiation capability and immunophenotypic. ADSCs were labeled with PKH-26during the 2nd passage **[7].**

Experimental protocol
Rats were divided into 4 main groups.

Control group (Group I)included fifteen rats. They were subdivided equally into three subgroups each included five rats. Negative control subgroup (Subgroup Ia): received regular diet and water only to determine the basic values for all used tests.Vehicle tap water subgroup(Subgroup Ib):received 1.0 ml tap water once a day through a stomach tube orally **[8].** Vehicle PBS subgroup(Subgroup Ic):the rats received 0.5ml of PBS in the tail vein **[9].**

Fluoxetine treated group (Group II) consisted of five rats that received 24 mg/kg/day of fluoxetine. It dissolved in 1.0 ml of tap water once a day through stomach tube. The animals were sacrificed after 30 days **[8].**

Fluoxetine group treated with ADSCs (Group III) consisted of five rats that received fluoxetine as group II for30 days then provide once with ADSCs at a dose of 1×10^6 cells/ rat extracted from male albino rats suspended in 0.5ml of PBS in the tail vein **[9].**Rats sacrificed 30 days after the stem cells injection.

Recovery group (Group IV) Include five rats that received fluoxetine for 30 days as group II then the animals received no treatment till ending the experiment. The rats were sacrificed 30 days after stopping fluoxetine treatment.

Histological and immunohistochemical study
Pars nervosa was excised and prepared for light and Immunohistochemical examination. Hematoxylin and eosin (H-E) stained sections were processed at 5 μm thickness. Immunohistochemical staining was performed for localization of S-100 protein (mainlynuclear and cytoplasmic in supporting cells). S-100 was done using the streptavidin-biotin immunoperoxidase method (streptavidin-biotin immunoperoxidase, Code No.ab52642, Dako).

Morphometric study
Done at the Faculty of Dentistry, Cairo University, Pathology Department using The Leica QWin 500 image analyzer (Leica Ltd). Supporting cells were assessed in S-100 immunostaining slides. The area percentage of S-100 distribution in immunostained slides was measured.

Statistical analysis
The data from image analyzer were analyzed using one-way analysis of variance (ANOVA) for comparison between groups (more than two groups). ANOVA test was statistically non-significant when P value >0.05, significant when P value <0.05 and highly significant when P value <0.001.

Results
Histological results
There were no significant differences in pars nervosa in the all control subgroups (Ia, Ib &Ic). They revealed nearly the same normal histological structures. So, their data were pooled together.

Examination of H-E–stained sections of the control rats revealed that the pars nervosa contained pituicyteswith oval to rounded nuclei and little cytoplasm which extend from nuclear regions to form short processes. Interspersed between these cells were capillaries. Also, Herring bodiesappeared as small islands of eosin stained substances**(Fig. 1A).**

Fluoxetine treated group showed major histopathological changes. H&E stained sections showed numerous pituicytes with irregular shaped nuclei and wide pale or vacuolated cytoplasm. Few Herring bodies were observed. Also, congested blood sinusoids and blood vessels with wide perivascular spaces were seen **(Fig. 1B).**

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Sections of Group III (Fluoxetine group treated with ADSCs) were almost similar to the normal microscopic appearance with minimal histopathological changes. Pars nervosa showed pituicytes with different shaped nucleiand pale cytoplasm. Capillaries are interspersed among these cells, and acidophilic small Herring bodies were noticed. Also, bi-nucleated cells could be seen in several sections **(Fig. 1C).**

Group IV (recovery group)showed more or less similar histological changes as those observed in Fluoxetine exposed group (group II). Pars nervosa showed some pituicytes with deeply stained nuclei, others had vesicular nuclei and pale or vacuolated cytoplasm. Few or ill-defined Herring bodies were observed. Also, congested blood sinusoids were identified**(Fig.1D).**

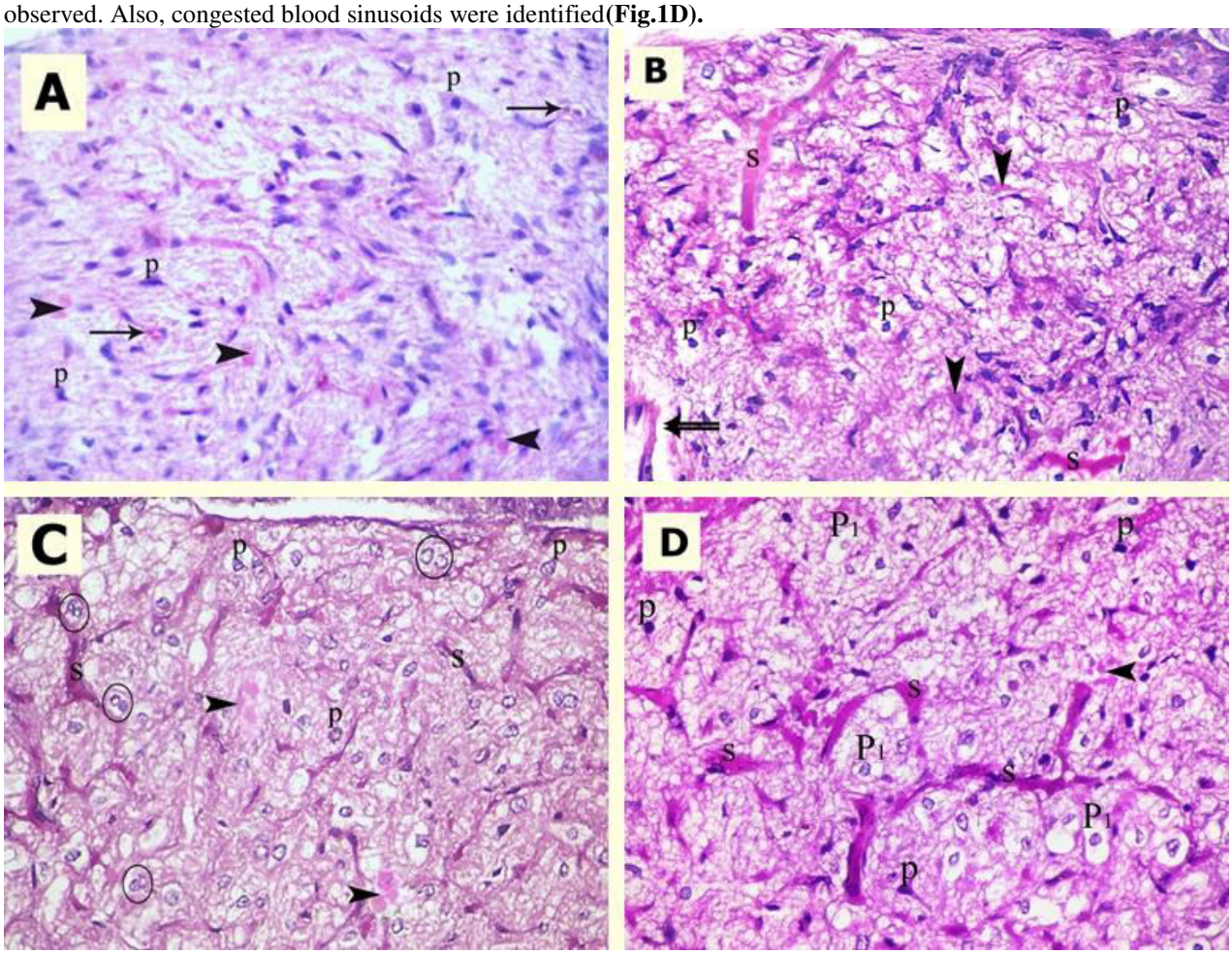

Figure 1. H-E–stained sections.**(A) Control group** showingpituicytes (P) with oval to rounded nuclei and little cytoplasm which extend from nuclear regions to form short processes. Capillaries (arrow) are interspersed between these cells. Also, Herring bodies (arrow head) appear as small islands of eosin stained substances. **(B) Fluoxetine treated group** showing numerous pituicytes (P) with irregular shaped nuclei and wide pale or vacuolated cytoplasm. Few Herring bodies (arrow head) can be noticed. Congested blood sinusoids (s) and blood vessel with a wide perivascular space can also be seen (double arrow). **(C) Fluoxetine treated with ADSCs**showing pituicytes (P) with different shaped nuclei and pale or vacuolated cytoplasm. Sinusoidal capillaries (s) are interspersed among these cells and also, acidophilic small Herring bodies (arrow head). Bi-nucleated cells (circle) are noticed. **(D) Recovery group**showing some pituicytes with deeply stained (P) and others with vesicular nuclei and pale or vacuolated cytoplasm (P1). Some Herring bodies (arrow head) are found. Congested blood sinusoids (s) are noticed.**(H &E, x400)**

Regarding S100 immunohistochemical analysis pars nervosa of the control group showed dispersed positive cytoplasmic immunoexpression pattern for S100 protein in the supporting pituicytes (**Fig. 2A**). Fluoxetine treated group revealed strong positive nuclear and cytoplasmic immunoexpression for S100 protein in the supporting pituicytes (**Fig. 2B**). Fluoxetine treated with ADSCs group (group III) exhibiting positive nuclear and cytoplasmic immunoexpression pattern for S100 protein in the supporting pituicytes(**Fig. 2C**).Recovery group showed wildlypositive nuclear and cytoplasmic

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immunoexpression for S100 protein in the supporting pituicytes (**Fig. 2D**).The area percent of S100was calculated in all groups **(Table I).**

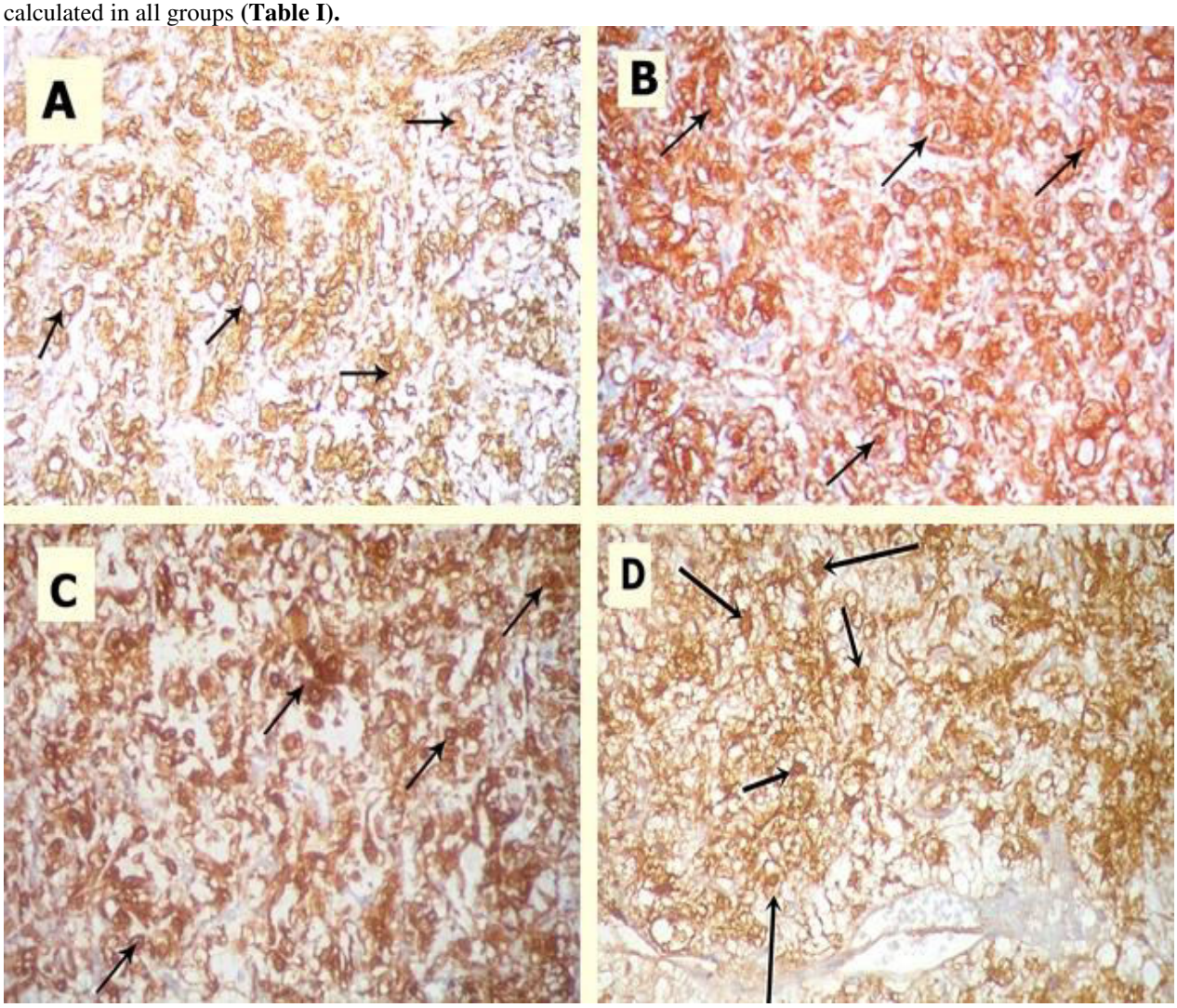

Figure 2. S100 immunostained sections. **(A) Control group** showingdispersed positive cytoplasmic and nuclearimmunoexpression for S100 protein in the supporting pituicytes (arrows). **(B) Fluoxetine treated group** showed strong positive nuclear, cytoplasmic immunoexpression pattern for S100 protein (arrows) in the supporting pituicytes.**(C) Fluoxetine treated with ADSCs**exhibiting positive nuclear and cytoplasmic immunoexpression pattern for S100 protein (arrows) in the supporting pituicytes. **(D) Recovery group** showing wildlypositive nuclear and cytoplasmic immunoexpression for S100 protein (arrows). **(S100 immunostain, x400).**

Morphometric results

Regarding the area percentage of S100 immunoreaction, statistical analysis of the morphometric results of the area percentage of supporting cells of pars nervosa showed high significant statistical difference when comparing group II (Flouxitine group) with group I (Control group). Whereas, there was significant difference when comparing group II (Flouxitine group) with group III (Flouxitine + ADSCs group) and group IV (Recovery group) **(Table I).**

Table (1): Comparison between mean values of S100 immune reaction in nucleus and cytoplasm of supporting cells of pars nervosa of adult male albino rats in the different studied groups using ANOVA (analysis of variance) test.

	Control group	Flouxitine group	Flouxitine + ASCs group	Recovery group	F	P value
S100 area % (pars nervosa)	13.57±2.02	17.74±1.20*	14.67±1.65 #	15.52±1.70 #	10.116	< 0.001

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		Control group	Flouxitine group	Flouxitine + ASCs group
S100 area % (pars nervosa)	Flouxitine group	< 0.001		
	Flouxitine + ASCs group	1.000	0.003	
	Recovery group	0.112	0.048	1.000

* Statistically high significant difference (p <0.001) when comparing group II (Flouxitine group) with group I (Control group).

\# Statistically significant difference (p> 0.05) when comparing group group II (Flouxitine group) with group III (Flouxitine + ADSCs group) and group IV (Recovery group).

Discussion

Depression is a psycho-physiological disorder involving feeling of sad-ness, despair and lack of energy. Fluoxetine is an antidepressant, easily available by the patients and approved for the treatment of major depression disorders (MDD) and panic disorder **[10].**

There have several reports for the effect of fluoxetine on hormonal axis of pituitary-gonad in rats. These studies have also shown that it has interaction with sex hormones in both sexes **[1].** Also, the histopathological effects of fluoxetine on vital organs; kidney and liver have been assessed **[3].** Most of the research that dealt with the exact structure pars nervosa was very old. However, it revealed very complex and scientific details that are believed to be vague to the most.

So, the current study was conducted to study the histological features of pars nervosa after chronic fluoxetine treatment and the possible recovery after cessation of this treatment.Also, this study was to evaluate the therapeutic effectiveness of ADSCs on the pars nervosa by analyzing the immunohistochemical.

In the current work, the most important observation noted was that most of the fluoxetine treated rats; numerous pituicytes with irregular shaped nuclei, pale or vacuolated cytoplasm and few Herring bodies were observed nearly in all sections. Also, blood vessels with wide perivascular spaces were seen.

In the recent study of **Faizal and Khan [11]** revealed that in the chronic hyperglycemia most of the pituicytes were darkly stained and quite many of them fell in the range of large-size. There was an overall increase in these darkly stained pituicytes of the rat. Therefore, **Lipinski [12] andYang et al. [13] reported** that one of the most important contributing factors in the development of pars nervosa dysfunction in chronic diabetes might be the hyperglycemia-induced neuronal cytotoxicity, pituicyte hyperplasia and hypertrophy. One of the possible explanations for the hypertrophy of pituicytes could be the increased production of hydrogen peroxide in glia following lipid peroxidation in all parts of the cell and thus affecting the membrane permeability in cytoplasmic organelles and nuclei. These results agreed with **Raasch et al. [14]** which indicated that fluoxetine stimulated the cellular changes by the same oxidative stress mechanism**.**

Our immunohistochemical findings revealed that the pituicytes had widely positive nuclear, cytoplasmic or cell membranes immunoexpression for S100 protein. Pituicyte like astrocyte, they are cellular residents of CNS involved in preserving the neuronal function and homeostasis and also like microglia, respond to numerous CNS insults. The experimental study of methotrexate treated rat frontal cortex displayed a significant increase in the area percentage of astrocytes glial fibrillary acidic protein (GFAP) immuno-expression or gliosis and these findings were similar to **Fardell et al. [15]** and **Feiock et al. [16]** and also supported our immunological study.

In the same context, quantitative image and statistical analysis showed that there was a highly significant increase in the positive S100 protein immunostaining area % of pituicytes supporting cells in fluoxetine treated group when compared with the control and while a significant increase to other groups.

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Pituicytes are commonly described as modified astroglial glial cells quite uniformly interspersed and lying in contact with the unmyelinated axons and Herring bodies. The main role of pituicytes is to assist in the storage and release of neurohypophysial hormones and it is the part of neurovascular component. These pituicytes interpose their processes between the secretory endings and basal lamina. Since any hormone that is secreted must pass through the basal lamina and into perivascular spaces in order to enter the fenestrated capillaries. Pituicyte interpositions form physical and perhaps chemical, barriers to hormone entering the circulation **[17]**.

In the present study, the administration of ADSCs led to marked or well improvement.The pars nervosa showednumerous pituicytes with variable shaped nuclei but few with pale cytoplasm. Acidophilic small Herring bodies and blood sinusoids were seen.

Stem cell therapy holds great promise for several lesions for its ability to self-renew, to differentiate into multiple lineages, to secrete neurotrophic factors and anti-inflammatory cytokines **[18].**

ADSCs might act by different possible mechanisms as previous mentioned; antioxidant, anti-apoptotic, anti-inflammatory. These cells also have the immune modulatory capacity to reduce inflammatory response and improve the microenvironment to promote the survival of both the transplanted and endogenous stem cells **[4].**

In addition, the differentiation into cells specific to the tissue; known as trans-differentiation and serve as an integrated member of the functionally organizing adult tissue was a mechanism. In several studies, this differentiation was taken strongly in consideration. For example, ADSCs play an important role in cardiovascular regeneration due to their ability to differentiate into a variety of cell lineages. Previous reports demonstrated that ADSCs were able to differentiate into cardiac muscle (CMs), endothelial cell (ECs) and vascular smooth muscle (VSMCs), which are the main components of the cardiovascular system **[19].**

We also noticed a significant decline in the mean is % of S100 positive expressing pituicytes cells. **Donega et al. [20]** and **Abdel khaleket al. [21]** recorded a similar result in brain mice and rat. After 15 and 28 days of MSCs treatment in ischemic brain and methotrexate treatment respectively, they noticed a decline in the amount of GFAP +ve expressing astrocytes which suggested the ability of MSCs to diminish gliosis. Another study by **Ribeiro et al. [22]** demonstrated that stem cells isolated from the adipose tissue and umbilical cord release trophic/neuroregulatory factors that improve the metabolic viability and neuronal with glial cell densities in primary cultures of hippocampal tissue.

In the current study, we had another attempt to reduce the fluoxetine harmful effects, which was to stop treatment for 30 days, and the results were somewhat unsatisfactory. This group revealed partial or minimal improvements after arrest or cessation of fluoxetine. Pars nervosa revealed some pituicytes with deeply stained nuclei, vesicular nuclei and pale or vacuolated cytoplasm. Few or ill-defined Herring bodies and congested blood sinusoids were observed in some sections.

Sobaniec- Lotowska [23] stated that 30 days after termination of chronic exposure to valproate, the abnormalities in hippocampus and neocortex did not subside. Whereas, after 90 days, may be features of distinct normalization could be observed.These findings may be supported by the study of **Csoka et al. [24]** who stated that symptoms of sexual dysfunction persist even after drug discontinuing. Persistence of some alterations after stopping fluoxetine treatment may indicate the need for more time for recovery or using appropriate protective drug.

In conclusion; our results revealed deleterious effects of fluoxetine on the histological structure of pars nervosa which probably may affect its functions in adult male rats. These histological changes could be alleviated by ADSCs treatment.

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