Fluoxetine induces direct inhibitory effects on mesenchymal stem cell-derived osteoprogenitor cells independent of serotonin concentration

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Abstract. Selective serotonin reuptake inhibitors are the most commonly prescribed antidepressants worldwide, which have been reported to exert potential detrimental effects on bone mineral density and increase the risk of developing fractures. The present study aimed to investigate the pathways underlying the negative effects of fluoxetine on bone using mesenchymal stem cells (MSCs) derived from rat adipose tissue as a source of osteoprogenitor cells. MSCs were harvested from adipose tissue using a collagenase enzyme digestion method and were allowed to differentiate into osteoprogenitor cells. Various concentrations of fluoxetine were added to the cells, which were harvested and analyzed by flow cytometry to detect apoptotic markers Annexin V and caspase-3, in order to assess the levels of apoptosis. The levels of endogenous serotonin released in the extracellular matrix were measured using a serotonin ELISA kit. The underlying molecular pathways associated with the effects of fluoxetine on bone were investigated with reverse transcription-quantitative polymerase chain reaction. The results of the present study revealed a significant dose-dependent increase in apoptosis in response to increasing doses of fluoxetine, which was independent of serotonin levels in the culture supernatant. These findings indicated that fluoxetine exerted a direct inhibitory effect on bone cells via an apoptosis-dependent pathway. Furthermore, the expression levels of serotonergic genes, including serotonin 1B receptor, serotonin 2A receptor (HTR2A), serotonin 2B receptor and serotonin transporter, were down regulated; of these genes, HTR2A exhibited the highest expression levels. Further *in vitro* and *in vivo* studies are required to verify this association and to determine the molecular pathways involved in fluoxetine-induced bone loss. Fluoxetine-induced apoptosis of osteoprogenitor cells may be the mechanism underlying the increased incidence of bone loss observed in patients treated with fluoxetine.

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Introduction

Selective serotonin/5-hydroxytryptamine (5-HT) reuptake inhibitors (SSRIs) are considered first-line therapy for the treatment of depression and account for ~60% of all antidepressant drugs prescribed worldwide (1). These therapeutic agents are prescribed for numerous psychiatric conditions, including post-traumatic stress, generalized anxiety, panic and premenstrual dysphoric disorders. In addition, they can be prescribed for certain non-psychiatric conditions, including chronic pain, fibromyalgia and post-menopausal vasomotor symptoms, such as night sweats and hot flashes (2). SSRIs act by antagonizing the 5-HT transporter (5HTT; also known as SLC6A4), enhancing the amount of 5-HT available in the synaptic cleft and potentiating serotonergic activity, thereby improving the symptoms of major depressive disorders (3).

A functional 5-HT signaling pathway was identified in bone in 2001 (4,5). A serotonin transporter has been detected in all major bone cell types, including osteoblasts, osteocytes and osteoclasts. Further investigation of this peripheral 5-HT system revealed that SSRIs affect the serotonin transporter in the central nervous system and bone with similar potency (6).

A dose-dependent increase in fracture risk and low bone mass has been reported to be associated with SSRI administration and the reason for these effects is unknown; however, direct and/or indirect 5-HT effects on bone cells may be the underlying cause (7). Osteoporosis results in a

biomechanically weakened skeleton; delays in bone healing resulting from medications, such as SSRIs, may increase the chance of prosthesis implant failure and may result in suboptimal outcomes (8).

The aim of the present study was to assess the mechanisms underlying SSRI fluoxetine toxicity on mesenchymal stem cells (MSCs) osteogenesis *in vitro* by measuring the concentration of serotonin expressed in osteoblasts following the administration of fluoxetine. In addition, the molecular pathways associated with the toxic effects of fluoxetine on bone cells were investigated by assessing the expression of specific genes. Additionally, the extent of apoptosis occurring in bone cells in response to various concentrations of fluoxetine was evaluated.

Materials and methods

Ethics statement and animals. The present study was conducted at the Medical Experimental Research Center (MERC), Faculty of Medicine, Mansoura University (Mansoura, Egypt). The protocol conducted in the present study was approved by the medical ethical committee of the Faculty of Medicine, Mansoura University.

Adipose tissue samples were collected from 12 male Sprague Dawley rats (6-8 weeks old, 250-280 g), which were purchased from the animal house at the MERC. The animals were housed at 24±2˚C, 60±10% relative humidity with a 12-h light/dark cycle. The rats were acclimated to the laboratory conditions, fed standard rat chow and water was available *ad libitum*. The tissues were collected under the supervision of a responsible veterinary doctor. The samples were collected in the operating room under complete aseptic conditions, and subcutaneous, intra-abdominal and peri-renal fat samples were obtained. The samples were preserved in sterile glass bottles containing PBS (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) and immediately transferred to the Stem Cell Laboratory (MERC) for processing.

Sample preparation procedures. Sample preparation was performed as described previously (9). The samples were washed numerous times with PBS supplemented with 10,000 units penicillin, 10 mg/ml streptomycin and 25 μg amphotericin B per ml. The samples were then sectioned into small pieces (1 mm^3) and treated with collagenase type I (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). An adequate amount of the enzyme (0.05% w/v) was added to the chopped fat tissue for 5 min and occasionally swirled in a water bath at 37˚C. The action of the enzyme was terminated using Dulbecco's modified Eagle's medium (DMEM)-low glucose media supplemented with 10% fetal bovine serum (FBS; both Sigma Aldrich; Merck KGaA) and 1% of penicillin/streptomycin/amphotericin; the suspension was then centrifuged at 200 x g and 4˚C for 10 min. Subsequently, the top oily layer was discarded and the remaining fluid was filtered through a 70-μm filter and centrifuged at 200 x g and 4˚C for 10 min. Cell viability was assessed using a Trypan blue exclusion assay. Cell viability was calculated as the number of viable cells divided by the total number of cells within the grids on the hemocytometer. If the cells took up trypan blue, they were considered non-viable. First the cell density of the cell line suspension was determined using a hemocytometer. Then, 0.4% solution of trypan blue in PBS was prepared, pH 7.2 to 7.3. A total of 0.1 ml trypan blue stock solution was added to 0.1 ml cells. The hemocytometer was loaded and the cells were examined immediately under light microscope at low magnification. Cell counting was done by counting the number of blue staining cells and the number of total cells. (% viable cells=[1.00-(Number of blue cells÷Number of total cells)] x100). The number of viable cells per ml culture was calculated using the formula below:

Number of viable cells $x10^4$x1.1=cells/ml culture and cell count was $1x10^6$ cells/mg tissue.

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-low glucose media (Sigma Aldrich; Merck KGaA) supplemented with 10% FBS, 10,000 U penicillin and 10 mg/ml streptomycin, in two 75-cm^2 tissue culture flasks and maintained in an incubator at 37˚C containing 5% CO_2. Cells were harvested once confluence reached 80% and cells from passage 3 were used for flow cytometric analysis.

As described by Zimmerlin *et al* (10), flow cytometric analysis was conducted to detect cellular expression of mouse anti-cluster of differentiation (CD)106 (cat. no. BBA5), anti-CD166 (cat. no. MAB6561), anti-CD146 (cat. no. MAB932), anti-CD105 (cat. no. MAB10971), anti-CD44 (cat. no. BBA10), anti-CD19 (cat. number MAB4867), anti-CD45 (cat. no. MAB1430), anti-CD90 (cat. no. MAB2067) and anti-Stro-1 (cat. no. MAB1038). The monoclonal antibodies (R&D Systems, Inc., Minneapolis, MN, USA) were conjugated to fluorescence isothiocyanate (FITC); for each marker, 90 μl of the cell suspension was added to 10 μl of antibody (dilution 1:10) and the cells were incubated for 30 min in dark at room temperature with the antibodies [Secondary developing reagent (cat. no. F0103B), Flow Cytometry Staining Buffer (R&D Systems, Inc.; cat. no. FC001) and isotype controls (R&D Systems, Inc.; cat. nos. MAB002 and MAB003; Caltag®; cat. no. MGM00]. Sterile PBS was used as a washing agent.

Osteogenic differentiation. Cells from passage 3 were seeded in 6-well plates at a density of $5x10^4$ cells/well. Following 24 h, the media were replaced with osteogenic media, which consisted of DMEM-low glucose media supplemented with 10% FBS, 100 units penicillin/ml, 100 mg streptomycin/ml, 10 mM b-glycerophosphate, 50 mg/ml 2-phosphate ascorbate and 10 nM dexamethasone (11). After 1 week, the cells were stained for calcium deposits using Alizarin red (Sigma Aldrich; Merck KGaA) for 30 min at room temperature in the dark. In addition to osteogenic differentiation, adipogenic differentiation was conducted to confirm multilineage differentiation potency of this population. Cells from passage 3 were seeded in 6-well plates at a density of $5x10^4$ cells/well. After 24 h, the media were replaced with adipogenic media, which consisted of DMEM-low glucose media supplemented with 10% FBS, with 10,000 units penicillin, 10 mg/ml streptomycin, 0.5 μmol/l isobutylmethylxanthine (1,000x0.5 mM in methanol), 50 μmol/l indomethacin (1,000x50 mM in methanol), and 0.5 μmol/l dexamethasone (1,000x0.5 mM in water; all from Sigma-Aldrich; Merck KGaA) (12). The medium was changed every 3 days; after 2 weeks, cytoplasmic oil droplets

were assessed via Oil Red O staining (20 min in dark at room temperature).

Drug application. Fluoxetine hydrochloride (Eli Lilly, Patheon France, France) was added to the media at the following concentrations: 0.5, 0.8, 1, 3, 5, 7, 10, 20 and 30 μmol/l for 5 days at 37˚C with 5% CO_2, and the media were replaced on days 0 and 3. Cells cultured in drug-free medium were considered the control group. Morphological alterations were observed at day 5 in all plates using an inverted microscope (Olympus Corporation, Tokyo, Japan) and the cells were analyzed by flow cytometry to detect the apoptotic markers Annexin V and caspase-3.

Detection of apoptosis

Annexin V analysis. A total of $1x10^5$ cells from all groups were stained with propidium iodide (PI) and FITC Annexin V (BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA). Briefly, 100 μl cell suspension was incubated with 5 μl FITC Annexin V and 5 μl PI in the dark for 15 min at room temperature; 400 μl binding buffer was added immediately prior to flow cytometric analysis using BD Accuri C6 software (BD Pharmingen; BD Biosciences).

Caspase-3 analysis. Briefly, the cells were washed twice with PBS, and $1x10^5$ cells from each group were incubated with 10 μl anti-caspase-3 conjugated to FITC (BD Pharmingen; BD Biosciences) in the dark for 20 min at room temperature. The cells were washed again with PBS, centrifuged for 5 min at 560 x g at 22-25˚C, stained with the FITC rabbit anti-active caspase-3 antibody for 30 min at room temperature, then washed, and suspended in BD Perm/Wash™ buffer (component of cat. no. 554714) before analyzing by flow cytometry Accuri C6 software (BD Pharmingen; BD Biosciences).

Quantitative assessment of serotonin. The levels of serotonin present in the groups treated with various concentrations of fluoxetine and in the control group were assessed using a\ serotonin ELISA kit (H0533; Glory Science Co., Ltd., Shanghai, China) according to the manufacturer's protocols. The culture supernatants were collected from the plates and preserved for ELISA. The samples were prepared by diluting 5-fold with a sample dilution reagent. The wells were covered with adhesive strips and incubated for 30 min at 37˚C. Following incubation, the adhesive strips were removed, the liquids (the sample tested and the standards for washing) were discarded, the wells were manually washed five times, each with the diluted wash solution and horseradish peroxidase was added to each well except the blank. Subsequently, the wells were incubated again for 30 min at 37˚C. Following incubation, the wells were washed again and 50 μl chromogen solution A and chromogen solution B were added to all wells and incubated for 15 min at 37˚C. The reaction was terminated by adding the stop solution, following which the color changed from blue to yellow. The color change was measured at 450 nm using a spectrophotometer. Data were calculated according to the following equation: $y=0.0115 x +0.1153$ and $R^2=0.9606$.

Assessment of serotonergic genes. Assays were conducted to quantify the expression levels of the following serotonergic receptors: Serotonin 1B receptor (HTR1B), serotonin 2A receptor (HTR2A), serotonin 2B receptor (HTR2B) and serotonin transporter (SLC6A4) under the effects of various drug concentrations of fluoxetine (1, 3, 5, 7, and 10 μmol/l). Total RNA was extracted from all samples after 5 days of differentiation using an RNA extraction kit (Promega Corporation, Madison, WI, USA). The concentration and quality of extracted RNA were assessed with a Nanophotometer P-330 (Implen GmbH, Munich, Germany). A total of 3 μg RNA was reverse transcribed using a SensiFast cDNA Synthesis kit (Bioline Reagents, Ltd., London, UK) according to the manufacturer's protocols. Quantification of mRNA expression levels was performed via reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using the SensiFast SYBR green system (Bioline Reagents, Ltd.). The reaction mixture contained 5 μl template cDNA with 1 μl (10 pM) forward and reverse primers of the specific genes, and 10 μl SYBR green premix, in a total volume of 20 μl. The thermocycling conditions were as follows: Denaturation at 95˚C for 2 min followed by 40 cycles of denaturation consisting of 5 sec at 95˚C and annealing for 30 sec at 55˚C for HTR1B, 60˚C for HTR2A, 53˚C for HTR2B, 53˚C for SLC6A4 and 58˚C for GAPDH and extension at 72˚C for 10 sec. The expression values were measured using the $2^{-\Delta\Delta Cq}$ method (13) and data were normalized to the loading control gene GAPDH. The primer sequences of the selected genes, as well as the loading control gene, were obtained from NCBI (https://www.ncbi.nlm.nih.gov/nuccore). The forward and reverse sequences are presented in Table I.

Statistical analysis. Data were tabulated and analyzed using SPSS version 23.0 (IBM Corp., Armonk, NY, USA). Data are presented as the mean ± standard deviation or the median and interquartile range (IQR). All experiments were performed once only. Significant differences were determined by one way analysis of variance, followed by a post-hoc Tukey test for parametric data, and a Kruskal Wallis test was performed for the analysis of non-parametric data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Rat MSCs isolation and characterization

Cells isolation. Rat MSCs were isolated from 10 g adipose tissue: $1x10^6$ cells/g. At passage 3, no major morphological alterations were observed during cell passaging. The primary and proliferating cells were fusiform in shape and exhibited fibroblast-like morphologies. The undifferentiated cells were observed to have small bodies with few cell processes, which were long and thin. The cell bodies contained a large, round nucleus with clear appearance (Fig. 1).

Cell characterization. Flow cytometric analysis of cells at passage 3 revealed that cells were positive for CD166, CD105, CD106, anti-Stro-1, CD146, CD44 and CD90. Conversely, the hematopoietic lineage markers, CD45 and CD19 were negative in >92% of cells (Fig. 2).

Multilineage differentiation for the determination of osteogenic differentiation. Cells from passage 3 to 7 were used for differentiation; calcium deposits and matrix mineralization

Table I. Primer sequences of serotonergic genes.

Gene	Forward	Reverse
HTR1B	5'-GATTGCCACAGTGTACCGGA-3'	5'-CAGGATGGACACAAGCAGGT-3
HTR2B	5'-CTCACTGGCTGCCTTCTTCA-3'	5'-GCGTTGAGGTGGCTTGTT TT-3'
HTR2A	5'-TCGTCATCATGGCAGTGTCC-3'	5'-ACAAGGAAACCCAGCAGCAT-3'
SLC6A4	5'-TTGGCTATGCTGTGGACCTG-3'	5'-TGATGGTGTAGGGGAGGAGG-3'
GAPDH	5'-CTCTGCTCCTCCTGTTCGAC-3'	5'-GCGCCCAATACGACCAAATC-3'

HTR1B, serotonin 1B receptor; HTR2A, serotonin 2A receptor; HTR2B, serotonin 2B receptor; SLC6A4, serotonin transporter.

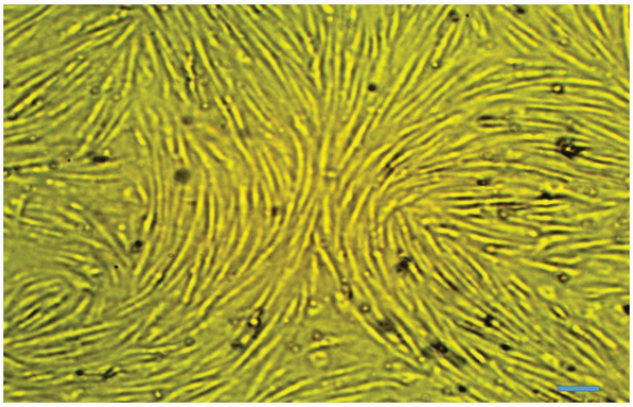

Figure 1. Rat mesenchymal stem cells exhibit fibroblast-like morphology. Cells were analyzed at 90% confluence and all populations appeared homogenous in shape. Scale bar, 20 μm.

were detected in the cytoplasm at day 5 using Alizarin red (Fig. 3A). For adipogenic differentiation, cells from passage 3 to 7 were used for differentiation; oil droplets were observed in the cytoplasm after 7 days of differentiation and were clear without staining, however; oil droplets became more evident when stained with Oil Red O (Fig. 3B and C).

Cell treatment with fluoxetine. Various morphological alterations were observed in the treatment groups: Shredding (loss of cellular adherence and integrity), absence of calcium deposits and cluster formations. These alterations were confirmed by flow cytometric analysis of the apoptotic markers caspase-3 and Annexin V.

Annexin V-FITC detection. There was a significant dose-dependent increase in apoptotic cells in response to increasing doses of fluoxetine. Highly significant late apoptosis was observed in cells treated with 20 μmol/l fluoxetine (45.2±6.1%). The lowest level of apoptosis was observed in cells treated with 5 μmol/l fluoxetine (17.5±3.7%). Data are presented in Fig. 4.

Caspase-3 detection. Anti-active caspase-3 antibodies were used to quantify the expression of caspase-3 in the osteoprogenitor cells under the effects of various concentrations of fluoxetine. The number of cells stained positive for caspase-3 was increased in a dose-dependent manner. The highest level of apoptosis was detected in cells treated with 30 μmol/l fluoxetine (22±4.5%). The lowest level of apoptosis was detected in cells treated with 5 μmol/l fluoxetine (10.6±2.3%). Data are presented in Fig. 5.

ELISA analysis of serotonin. The amount of serotonin in the culture supernatants was measured at day 5 for all fluoxetine-treated groups (1, 3, 5, 7 and 10 μmol/l fluoxetine) and the control group. Data were expressed as the median and IQR, and a Kruskal Wallis test was used to determine significance (Table II). There was no significant difference in extracellular serotonin concentrations in response to increasing doses of fluoxetine when compared with the control group (P=0.098).

RT-qPCR analysis of serotonergic genes. The results of RT-qPCR analysis demonstrated that a statistically significant dose-dependent increase in the expression levels of serotonergic receptors, including HTR1B, HTR2A and HTR2B, as well as the serotonin transporter SLC6A4, was detected in osteoprogenitor cells in response to increasing doses of fluoxetine. The expression levels of all receptors were increased in response to 10 μmol/fluoxetine and the lowest levels of expression were reported in cells treated with 3 μmol/fluoxetine; however, all expression levels were significantly lower compared with the control group (P<0.001; Fig. 6). These results suggested that rat bone cells may exhibit higher expression levels of HTR2A compared with HTR1B and HTR2B in response to fluoxetine.

Discussion

SSRIs are the most common type of drug used to treat psychiatric disorders and are the first line treatment for major depressive and anxiety disorders (14). Several *in vitro* studies have reported the presence of serotonin receptors in primary bone cells and/or bone cell lines, which mainly harbor HTR1A, HTR2A and HTR2B binding sites (4,5). Conflicting data have been reported regarding the effects of serotonin and SSRIs on bone; both beneficial and detrimental effects have been observed (15).

In the present study, the effects of fluoxetine on osteoprogenitor cells were investigated using rat adipose tissue-derived MSCs as a source of osteoprogenitor cells. In depth analysis of the effects of fluoxetine on bone cells *in vitro* was conducted using histochemical analysis with Alizarin red staining. Subsequently, flow cytometric detection of the extent of cell apoptosis was conducted using Annexin V and caspase-3 markers, and the expression levels of serotonin in the cell supernatant were detected in response to various concentrations of

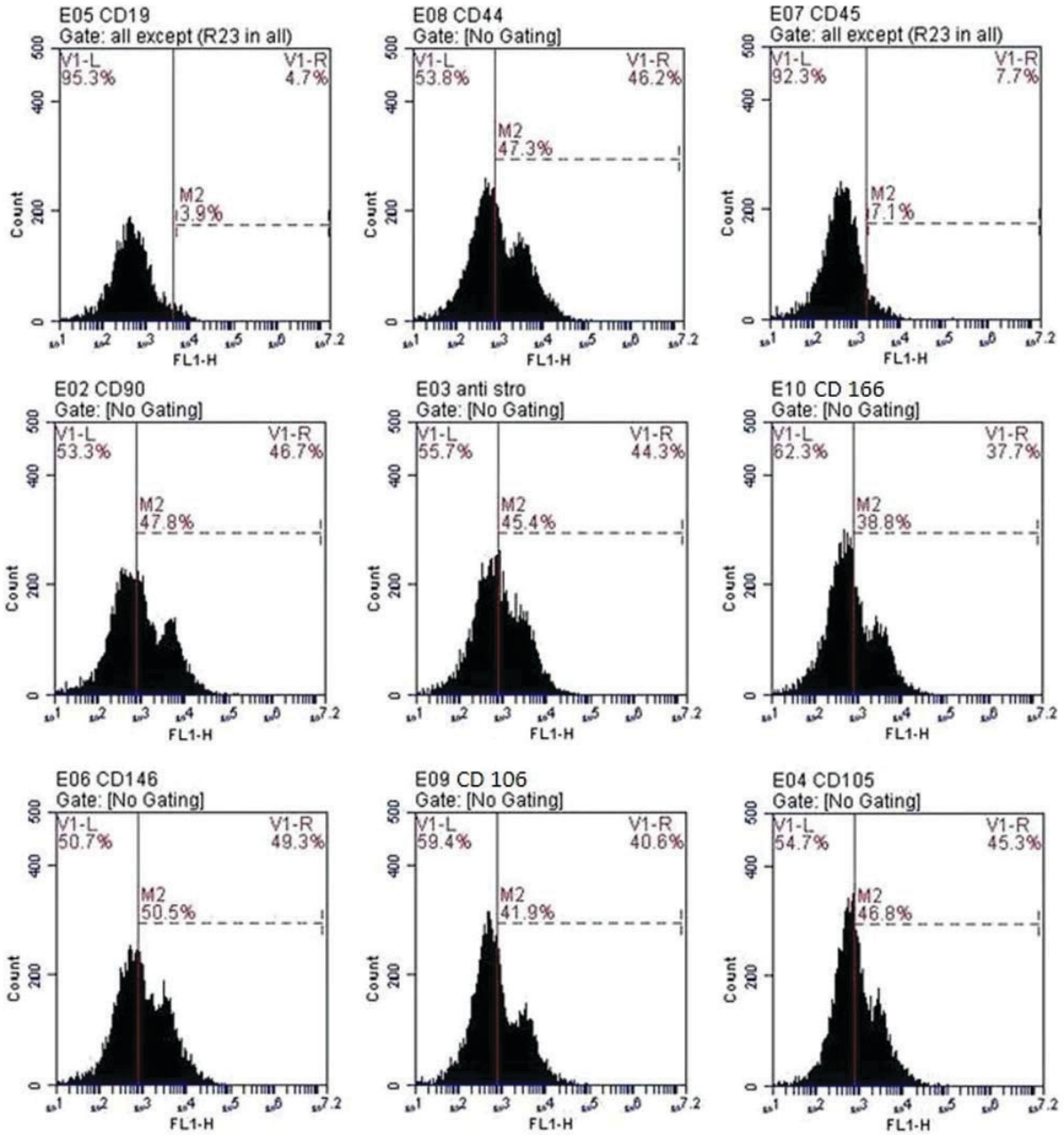

Figure 2. Flow cytometric analysis of rat MSCs. MSCs were positive for CD166, CD105, CD106, anti-Stro-1, CD146, CD44 and CD90, and negative for the hematopoietic markers CD45 and CD19. CD, cluster of differentiation; MSC, mesenchymal stem cell.

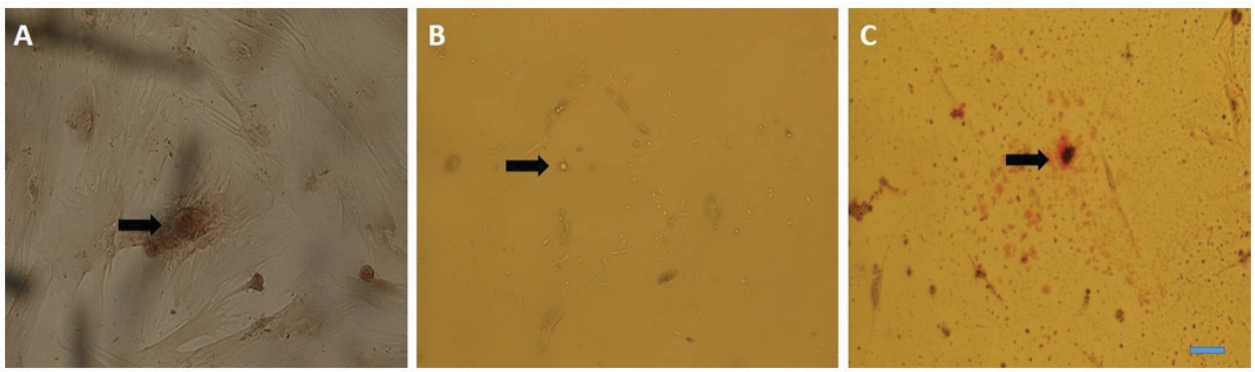

Figure 3. Multilineage differentiation of rat mesenchymal stem cells. (A) Alizarin red staining revealed evident extracellular matrix mineralization and formation of calcium clusters (arrow). (B) Stromal cells following the induction of adipogenic differentiation with evident oil droplets (arrow). (C) Oil red O staining revealed evident oil droplets (arrow) in the extracellular matrix. Scale bar, 20 μm.

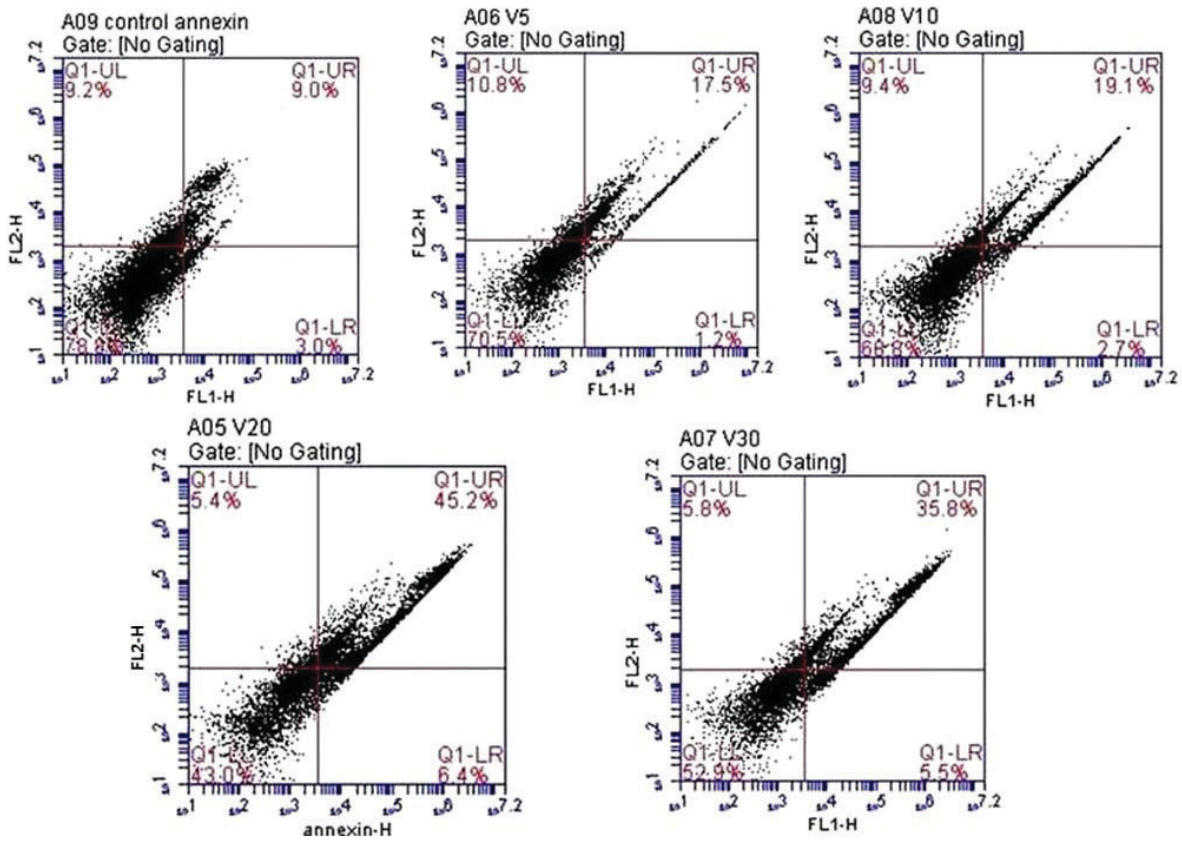

Figure 4. Flow cytometric analysis of apoptosis using Annexin V. V5 refers to 5 μmol/l, V10 refers to 10 μmol/l, V20 refers to 20 μmol/l and V30 refers to 30 μmol/l fluoxetine. The highest level of apoptosis was detected in cells treated with 20 μmol/l fluoxetine.

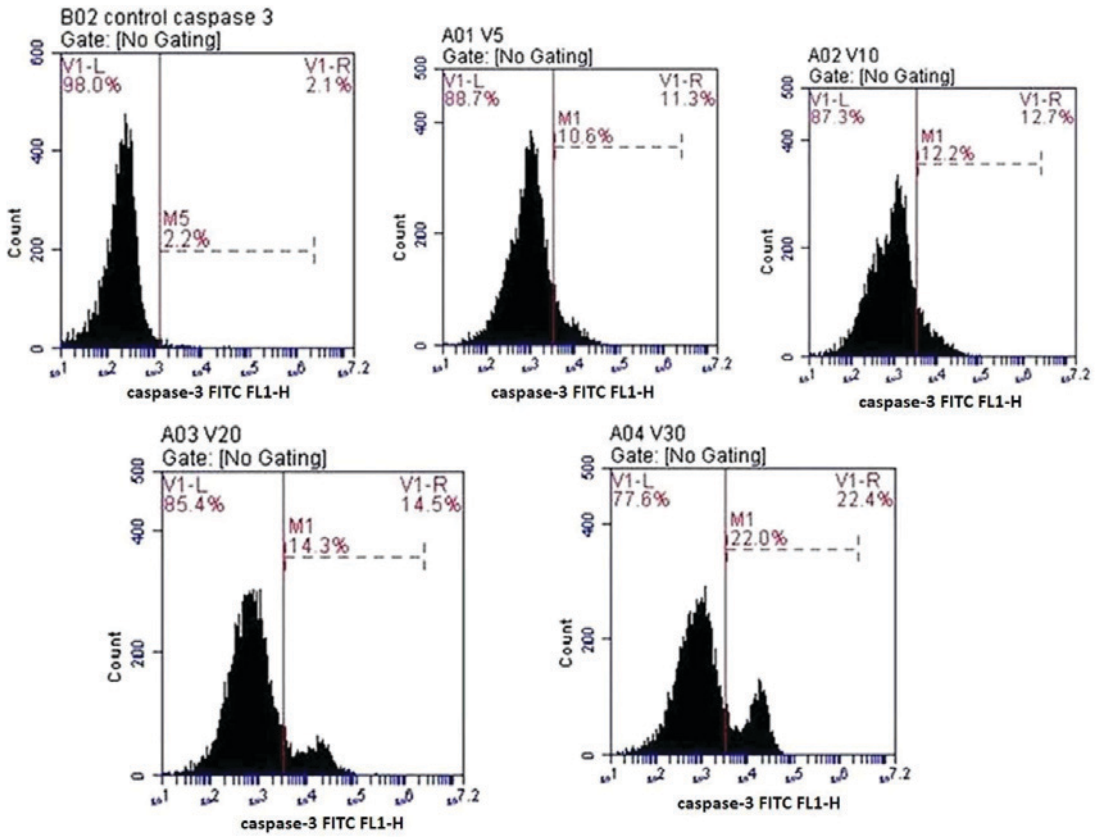

Figure 5. Flow cytometric analysis of apoptosis using caspase-3. V5 refers to 5 μmol/l, V10 refers to 10 μmol/l, V20 refers to 20 μmol/l and V30 refers to 30 μmol/l fluoxetine. The highest level of apoptosis was detected in cells treated with 30 μmol/l fluoxetine. FITC, fluorescein isothiocyanate.

Table II. Comparison between serotonin concentrations and increasing doses of fluoxetine.

	Control group	1 μmol/l group	3 μmol/l group	5 μmol/l group	7 μmol/l group	10 μmol/l group	P-value
Median	15.93	29.45	15.89	15.15	19.94	58	0.098
IQR	7.082	23.63	8.941	8.256	11.06	8.800	
	22.73	42.80	69.34	18.80	22.80	18.80	

IQR, interquartile range.

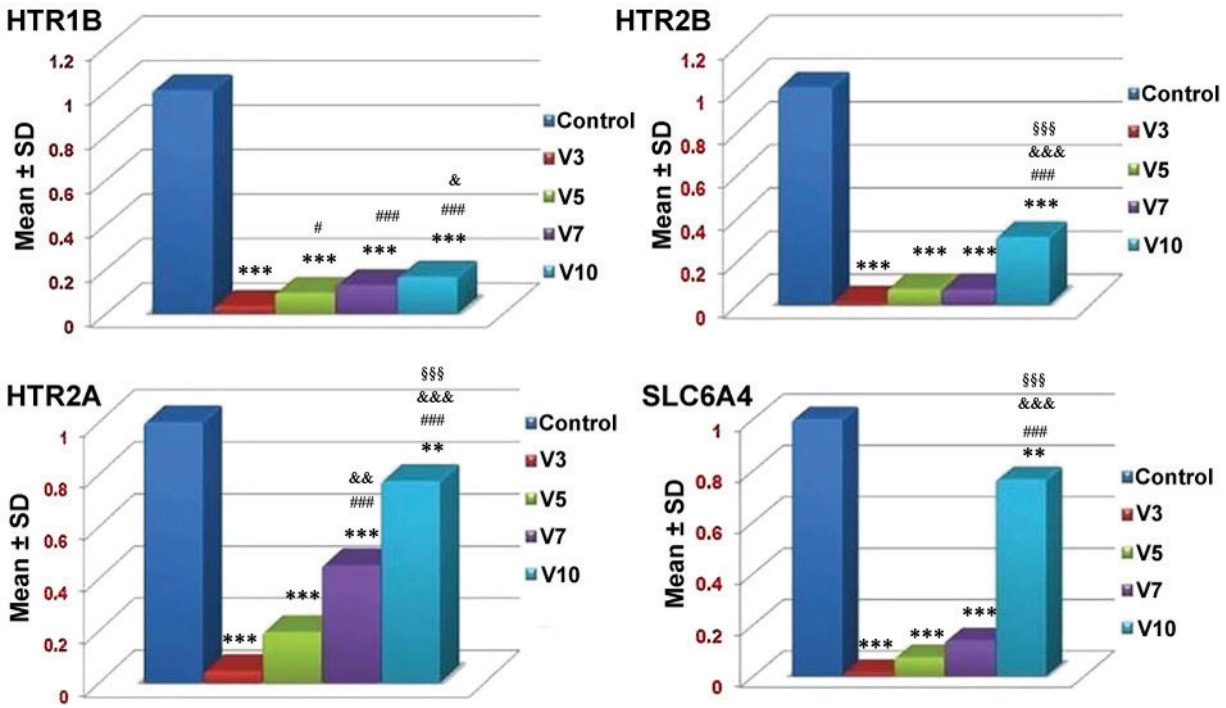

Figure 6. mRNA expression levels of HTR1B, HTR2B, HTR2A and SLC6A4 receptors in the control and fluoxetine-treated groups. Fluoxetine downregulated all investigated serotonergic genes and SLC6A4. V3 refers to 3 μmol/l fluoxetine, V5 refers to 5 μmol/l fluoxetine, V7 refers to 7 μmol/l fluoxetine and V10 refers to 10 μmol/l fluoxetine. **P<0.01, ***P<0.001, vs. the control. $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. V3. $^{\&}$P<0.05, $^{\&\&\&}$P<0.001 vs. V5. §§§P<0.001 vs. V7. HTR1B, serotonin 1B receptor; HTR2A, serotonin 2A receptor; HTR2B, serotonin 2B receptor; SD, standard deviation; SLC6A4, serotonin transporter.

fluoxetine using ELISA. Additionally, RT-qPCR analysis of serotonergic genes was performed to elucidate the underlying molecular pathways associated with the effects of fluoxetine on osteoprogenitor cells.

The results of the present study demonstrated that fluoxetine inhibited osteoprogenitor cell proliferation, differentiation and mineralization in a dose-dependent manner, which may occur via the apoptotic pathway alongside downregulated expression levels of serotonin-associated genes independent of serotonin concentration. Various morphological alterations were reported in cells in response to increasing doses of fluoxetine. Flow cytometric analysis of apoptosis using Annexin V and caspasee-3 markers supported these morphological alterations; increased levels of apoptosis were associated with increasing doses of fluoxetine in the present study. A concentration of 20 μmol/l was associated with cytotoxicity; the proportion of apoptotic cells was ~45.5% by annexin staining. The present study observed that this cytotoxic effect may be independent of the levels of serotonin within cells. Analysis of intracellular serotonin levels following exposure to various concentrations of fluoxetine indicated that there was an insignificant association between drug dose and the levels of serotonin released. This result supported the hypothesis that apoptosis may be the mechanism underlying the detrimental effects of fluoxetine on osteoprogenitor cells. RT-qPCR analysis detected a significant dose-dependent downregulation of all serotonergic genes, including HTR1A, HTR2A and HTR2B, and SLC6A4. The expression of the HTR2A receptor was the most significantly affected within all treated groups, suggesting an important role of these G-protein coupled receptors on growing bone cells which might need further research work.

The findings of the present study are consistent with those of Hodge *et al* (16), who conducted a systematic *in vitro* study investigating the effect of fluoxetine and other SSRIs on trabecular bone cells. In addition, it was reported that treatment of human osteoblasts with increasing doses of exogenous serotonin (3-30 μmol/l) exhibits no effect on bone mineralization or alkaline phosphatase activity (16). However, Ortuño *et al* (17) revealed the inhibitory effect of fluoxetine on developing bone cells; alternative molecular pathways

were suggested (17). Ortuño *et al* (17) identified a dual role of fluoxetine on bone remodeling, in which short-term (3 weeks) treatment with fluoxetine was revealed to result in a local anti-resorptive response that increases bone mass, directly impairing osteoclast differentiation and function via a serotonin reuptake-independent Ca^{2+}-calmodulin-nuclear factor of activated T cells 1-dependent mechanisms. However, chronic treatment (6 weeks) with fluoxetine was revealed to induce a central serotonin-dependent increase in sympathetic output flow, which results in increased bone resorption sufficient to counteract the local anti-resorptive effects, therefore leading to a net effect of decreased bone formation and bone loss (17).

The present study proposed that fluoxetine induces apoptosis in differentiating bone cells and reduces osteoblasts proliferation (as tested by measuring cell viability), mineralization of the bone cells and the formation of new healthy cells; some studies have indicated that fluoxetine not only inhibits the activity of serotonin but also directly affects cell proliferation and apoptosis in other body tissues: Fluoxetine has been reported to induce apoptosis of the hippocampus and cortical neuronal cells within developing rats as demonstrated by Schaz *et al* (18).

A recent *in vivo* study conducted by Rafiei *et al* (19) investigated the effects of fluoxetine on maxillary teeth in rats by administering fluoxetine (10 mg/kg) intraperitoneally 5 times/week for 1 month. The results indicated that fluoxetine exerts an inhibitory effect on osteoprogenitor cell regeneration (19). The findings of the present study regarding the effects of fluoxetine on serotonergic genes were consistent with those of Gustafsson *et al* (20), who reported that stimulation of HTR2A receptors results in reduced signaling for differentiation within osteoblasts. Additionally, it has been reported that the expression levels of HTR2A were higher compared with HTR2B within rat bone cells (21,22). In the present work, it was demonstrated that fluoxetine may have a direct inhibitory effect on the expression of this receptor. To investigate this theory regarding the effect of fluoxetine, more research regarding this point is necessary.

Compared with the findings of the present study, previous reports have demonstrated positive anabolic effects of fluoxetine on bone cells. Battaglino *et al* (23) demonstrated that treatment of Swiss-Webster mice with fluoxetine at a dose of 10 mg/kg/d may stimulate bone formation in the femur and lumbar vertebrae. Mortazavi *et al* (24) reported an increase in bone formation following treatment with fluoxetine of rats with calvarial small-size bone defects. A possible explanation for this difference in results may be the variation in the fluoxetine treatment protocol, using different strains of animals or genetic differences in the origin of the tested cells. The results of Nam *et al* (22) were in accordance with those of the present study regarding the inhibitory effects of fluoxetine on bone cells, however; serotonin was proposed to exhibit a direct inhibitory effect on osteoprogenitor cells (22), contradictory to the results of the present study.

In conclusion, the present study demonstrated a direct inhibitory effect of fluoxetine on bone cells, which may be dependent on apoptosis rather than on serotonin levels. Additionally, there was an overall down regulation in the expression of serotonin receptors HTR1B, HTR2A, HTR2B as well as 5HTTT (SLC6A4) in response to varying doses of fluoxetine. The findings of the present study may indicate that fluoxetine consumption negatively affects bone mineral density with the possible increase in the rate of bone fracture.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MS, AL and MAS designed the study; SMK, MS and MEH performed the experiments; SMK, MS, MEH, MESAK, AL, SAH, SAGE and MAS performed data analysis; and, SMK, MS and MEH wrote the manuscript.

Ethics approval and consent to participate

The protocol conducted in the present study was approved by the medical ethical committee of the Faculty of Medicine, Mansoura University (Mansoura, Egypt).

Patient consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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